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Quality Assessment and Quantitative Analysis of Flavonoids from Tea Samples of Different Origins by HPLC-DAD-ESI-MS

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Components of green tea (*Camellia sinensis*) have been of considerable interest in recent years because of their potential utility as pharmaceutical agents, particularly for their antioxidant and anticarcinogenic activity. Responding to the increasing scientific validation of numerous health benefits of tea, a comprehensive approach was adopted to carry out analysis for the quality assessment of flavonoids in tea samples of different origins. For this purpose, extraction, separation, and mass spectrometric parameters were optimized. Extraction methods evaluated include reflux extraction, a modified accelerated solvent extraction (ASE), namely, Aquasolv extraction, and microwave-assisted extraction (MAE) using different percentages of solvents. Separation was performed by a specifically developed reversed phase high-performance liquid chromatography (RP-HPLC) method using different C18 and C8 stationary phases. Optimization of extraction techniques clearly proved the performance of MAE, which delivered highest yields in a very short time. Additionally, the comparison with Aquasolv extraction provided new insights, as variations in quantified amounts of target compounds between the extracts could be explained on the basis of thermal degradation and epimerization phenomena. Especially the epimerization phenomenon for catechin/epicatechin oligomers, that is, of procyanidins P_2 and P_3, was observed for the first time. Finally, an optimized extraction and separation system was used for qualitative and quantitative investigations of compounds from different green tea samples from Ceylon (cultivated under biologically controlled conditions), Japan, India, and China as well as from one black tea sample from India.

KEYWORDS: Green tea; extraction; epimerization; catechin; epicatechin; microwave-assisted extraction

INTRODUCTION

Tea is one of the most widely consumed beverages and is grown in 30 countries worldwide. The tea plant (*Camellia sinensis* L. Theaceae) originated in southeastern China. Approximately 3 million metric tons of dried tea is produced annually as green tea (20%), oolong tea (2%), and black tea (78%) (*1*). The chemical composition of tea depends on several factors such as genetic strain, climatic conditions, horticultural practices, soil, growth altitude, plucking season, sorting (grading) of the leaves, processing, and, in the case of tea extracts, the conditions and technologies used for extraction, storage, and drying (*2, 3*).

Green tea antioxidants have drawn increased attention and considerable interest during the past decade because of the potential utility of some of the compounds as pharmaceutical agents, finding applications in a wide range of products, for example, foods, beverages, toiletries, and cosmetics (*4, 5*). It is particularly important for quality evaluation not only in relation to its antihypertensive, antioxidative, and hypolipidemic properties but also as an anticarcinogenic, antitumorigenic, antiarteriosclerotic, and antimicrobial agent (*6, 7*). It has a marked influence in reducing the risk of chronic diseases, promoting oral health, and prolonging the shelf life of food products without damage to their organoleptic or nutritional qualities. Moreover, it plays a vital protective role against cardiovascular diseases, effectively scavenges superoxide free radicals and hydroxyl radicals, and prevents Cu-mediated LDL oxidation (*2, 8–10*).

These effects are mostly attributed to tea polyphenols consisting mainly of flavonols and flavan-3-ols commonly known as catechins, including also their gallate derivatives. The growing interest in the latter derives from their strong activity and low toxicity as compared to synthetic phenolic antioxidants.

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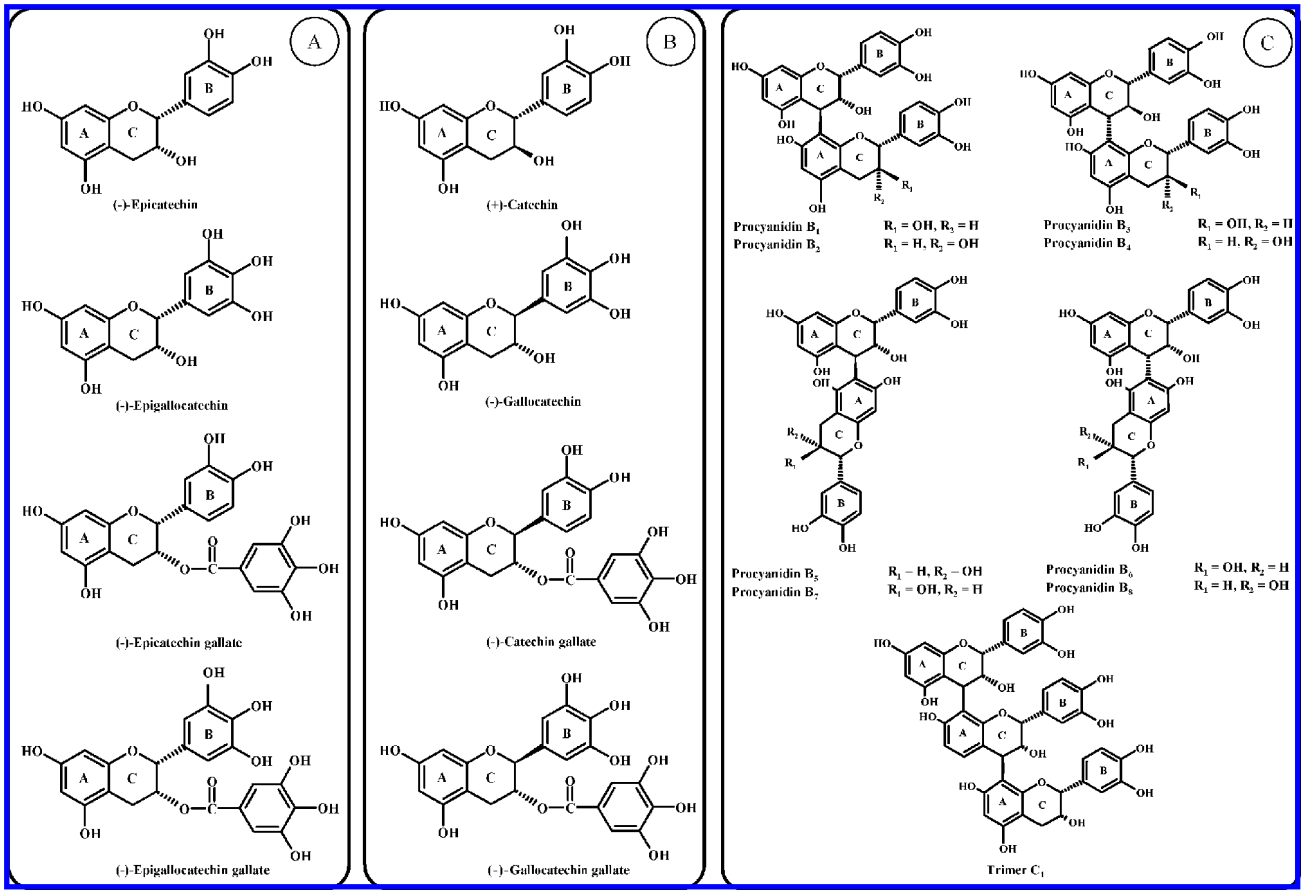

Figure 1. Chemical structures of investigated (**A**) epicatechins, (**B**) catechins, and (**C**) oligomeric procyanidins.

The structures, pharmacokinetics, and bioactivities of the conjugated forms of tea catechins and all of their metabolites are not yet fully known (*11*). Flavan-3-ol monomers contribute 20–25% to the dry weight of tea leaves (*12*). The main flavonols in tea are conjugates of quercetin and kaempferol and lower levels of myricetin with mono-, di-, tri-, and sometimes tetrasaccharides forming multiple glycosides (*1*). In black tea, approximately 10% of the flavonoids are catechins, 10% are theaflavins, and 70% are thearubigins (*13*). Proanthocyandins are polymeric flavanols, which are present in plants as complex mixtures of polymers with an average degree of polymerization between 4 and 11. They are responsible for the astringency of food (*7, 14*).

Generally, different techniques have been published concerning the development of extraction procedures (*15*). Very simple methods such as maceration, percolation, digestion, reflux extraction (RE), Soxhlet extraction (SE), and ultrasonic extraction (UE) are available in addition to more exhaustive and sophisticated techniques such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), and several other modifications thereof. Liu et al. (*16*) published the comparison of flavonoid extraction yields obtained from *Epimedium koreamum* by pressurized microwave-assisted extraction (PMAE), atmospheric pressure microwave-assisted extraction (AMAE), UE, and RE. They obtained highest yields using PMAE and lowest with UE. Gao et al. (*17*) compared the extraction efficiency of different methods for flavonoids from *Saussurea medusa*, for example, room temperature extraction, heat reflux extraction, SE, UE, and MAE. MAE showed obvious advantages over all other methods in short time duration and high extraction efficiency. In the case of green tea, Pan et al. (*18*) found in agreement with Liu et al. (*16*) that MAE was more effective than other conventional extraction methods such as extraction at room temperature, UE, and heat reflux extraction.

Chen et al. (*6*) studied the influence of the extraction technique on the stability of catechin and catechin derivatives, which are analytes present in high abundance in green tea. By autoclaving the water extract, they found only 76% of catechin derivatives. Wang et al. (*19*) found no change in the sample when extracting at room temperature or at 40 °C (sample in water), but significant conversion from epicatechin to catechin at 80 °C and further accelerated effects when using tap water (*10*) and longer duration of extraction. Such epimerization phenomena occurring during the extraction procedure were also studied by Xu et al. (*9, 20*), who found that epimers were not originally present in green tea, but were produced by a thermally induced epimerization reaction. Finally, the same group published also the occurrence of degradation besides epimerization effects (*6, 20*).

Most of the chromatographic techniques developed so far have been aimed at characterizing catechins and other compounds believed to be possible quality markers, for example, proanthocyanidins (*7*) and other flavonoids (*2*). In fact, various techniques have been used for the determination of these phenolic compounds, but prime interest was given to chromatographic methods such as high-performance liquid chromatography−UV detection (HPLC-UV) (*3, 6, 21–23*) and most abundantly HPLC-MS (*3, 8, 16, 24–31*). Recent studies on the characterization and identification of individual components of complex plant extracts and on the characterization of polyphenolic compounds from green tea have shown that especially HPLC-ESI-MS is a powerful tool for structure elucidation (*5*).

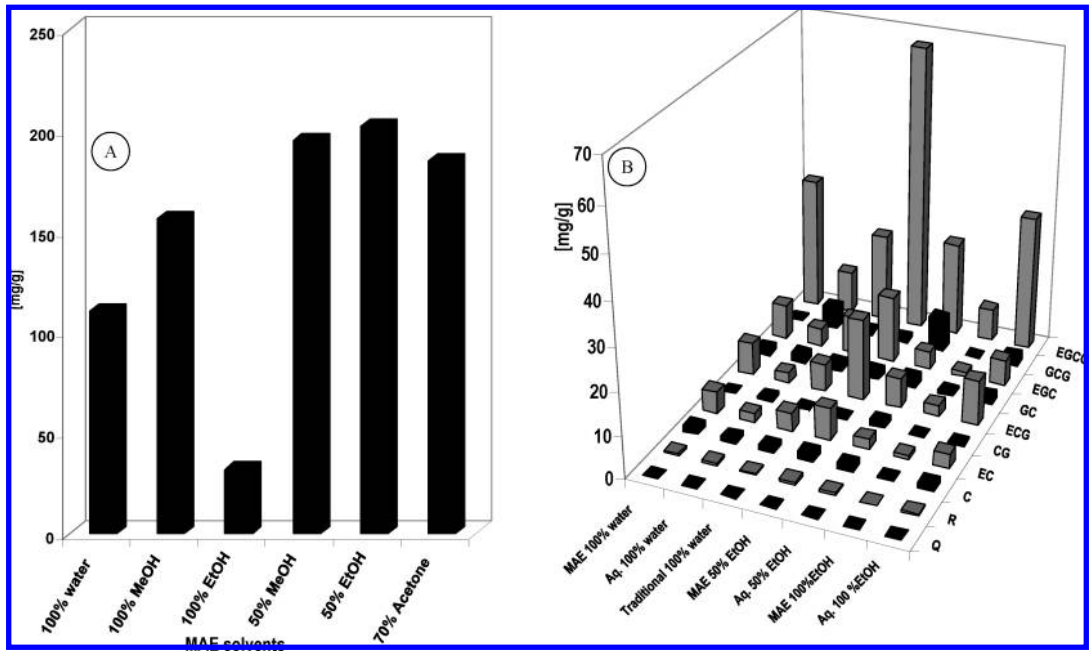

Figure 2. (**A**) Optimization of solvent extraction using MAE and Folin−Ciocalteu reagent for quantification. (**B**) Quantified amounts for different flavonoids in 1 g of green tea sample extracted by different methods. Aq, Aquasolv extraction; MAE, microwave assisted extraction; C, catechin; EC, epicatechin; CG, catechin gallate; ECG, epicatechin gallate; GC, gallocatechin; EGC, epigallocatechin; GCG, gallocatechin gallate; EGCG, epigallocatechin gallate; Q, quercitrin; R, rutin; sample = green tea 48 India.

Table 1. Evaluation of Different Techniques for the Extraction of TTP[a]

		extract (%)		TTP (%)	
sample ID	sample name	tap water	distilled water	tap water	distilled water
	Traditional Extraction				
1	traditional GT 48 India	28.5	29.9	12.7	15.5
2	traditional GT 53 Japan	21.9	23.7	6.2	5.9
3	traditional GT 60 China	27.0	25.1	9.4	11.7
4	traditional GT 68 Ceylon	27.3	28.8	15.1	15.0
5	traditional BT 7 India	24.9	25.5	11.3	11.1
6	traditional BT Tetley	24.3	22.5	6.8	8.1
7	traditional GT Teefix	28.1	25.5	11.7	10.3
	Microwave Assisted Extraction				
1	MAE GT 48 India	30.2	22.2	16.1	10.1
2	MAE GT 53 Japan	22.9	23.0	9.1	9.5
3	MAE GT 60 China	27.4	25.8	11.1	11.5
4	MAE GT 68 Ceylon	29.1	28.3	9.7	14.6
5	MAE BT 7 India	26.9	24.9	11.5	10.6
6	MAE BT Tetley	22.2	21.0	8.1	6.9
7	MAE GT Teefix	27.7	26.5	10.7	10.9
	Aquasolv Extraction				
1	Aquasolv GT 48 India	24.7	26.1	11.3	13.7
2	Aquasolv GT 53 Japan	14.6	20.6	4.4	8.1
3	Aquasolv GT 60 China	19.5	19.8	5.6	7.4
4	Aquasolv GT 68 Ceylon	18.3	25.7	11.2	11.9
5	Aquasolv BT 7 India	23.7	23.4	5.3	8.3
6	Aquasolv BT Tetley	12.3	9.3	2.7	2.9
7	Aquasolv GT Teefix	N.M	21.2	N.M	8.1

[a] Data are expressed as mean ($n = 2$). TTP, total tea polyphenols; extract (%), percentage of dry wt of extract to dry wt of tea; TTP (%), percentage of TTP to dry wt of tea; GT, green tea; BT, black tea; MAE, microwave assisted extraction; NM, not measured.

The aim of this study was the evaluation of different extraction methods, especially a relatively new extraction technique, that is, Aquasolv extraction. Special regard was given to the epimerization phenomenon occurring during the extraction procedure in the case of catechin monomers and corresponding oligomers (procyanidins). Finally, selected flavonoids in green tea samples were quantified.

MATERIALS AND METHODS

Chemicals and Reagents. (+)-Catechin (hydrate, ≥98%), (−)-epicatechin, (−)-epigallocatechin (HPLC, ≥98%), (−)-epicatechin gallate (HPLC, ≥98%), (−)-epigallocatechin gallate (HPLC, ≥80%), quercetin dihydrate (HPLC minimum, 98%), gallic acid, rutin hydrate (95%), quercitrin hydrate (85%), Folin−Ciocalteu's phenol reagent, ethanol (absolute, 99.8%), acetonitrile (gradient grade), methanol (gradient grade), and sodium carbonate anhydrous were purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid and 1-propanol were supplied from Merck (Darmstadt, Germany). Water was used after purification by an Infinity Nanopure unit (Barnstead, Boston, MA). Tea samples of different origins, for example, green tea *48 India* (Assam, India), green tea *60 China* ("Temple of heaven", China), green tea *68 Ceylon* (cultivated under biologically controlled conditions), green tea *53 Japan* (Sencha "Fukujyu", Japan), and black tea *7 India* (*Darjeeling SF* "Highland", India) were purchased from a specialty tea shop (Rauter Tee, Innsbruck, Austria). On the basis of the information given by the supplier, all above-mentioned tea samples were considered to be of comparable quality. Commercial tea samples such as green tea "Teefix" and black tea "Tetley" were chosen from a local grocery store.

Sample Preparation. *Standards.* Stock solutions of standards were prepared in the concentration range from 1.8 to 2.6 mg/mL in ethanol/water (50:50, v/v) and stored at –20 °C until use. Standard working solutions were prepared by diluting stock solutions to desired concentrations (0.5 μg/mL–1.6 mg/mL). Five-point calibration curves were established for quantification, each of which was measured three times.

To carry out reliability tests, standard solutions of gallic acid (0.1 mg/mL) and quercetin (0.05 mg/mL) were prepared in ethanol/water (50:50, v/v), methanol/water (50:50, v/v), and 1-propanol/water (50:50, v/v).

Solubilization and Extraction. Microwave-assisted extraction (MAE), Aquasolv extraction, and reflux extraction were performed. After extraction, samples were frozen at –20 °C in darkness until use.

Traditional Reflux Extraction (TE). The traditional extraction of different samples was carried out using a carousel reaction station (Radleys Saffron Walden, U.K.) equipped with a water reflux cooling

Table 2. Quantitative Determination of Total Catechins (TC), Total Epicatechins (TEC), Total Catechin Derivatives (TCD), and Total Polyphenols (TTP) in Tea Samples of Different Origins[a]

sample name	(%) TC/TCD (w/w)	(%) TEC/TCD (w/w)	(%) TC/TTP (w/w)	(%) TEC/TTP (w/w)	(%) TCD/TTP (w/w)	(%) TC/dry wt (w/w)	(%) TEC/dry wt (w/w)	(%) TCD/dry wt (w/w)	(%) TTP/dry wt (w/w)
MAE GT 48 India	4.3	95.7	3.7	83.7	87.5	0.5	11.0	11.5	13.1
MAE GT 53 Japan	2.1	97.9	1.9	88.3	90.1	0.2	10.4	10.6	11.7
MAE GT 60 China	2.0	98.1	1.6	77.9	79.4	0.2	10.7	10.9	13.7
MAE GT 68 Ceylon	4.5	95.5	3.7	76.9	80.6	0.6	12.2	12.8	15.9
MAE BT 7 India	2.9	97.1	0.9	31.5	32.5	0.1	4.0	4.1	12.7
AE GT 48 India	26.6	73.4	9.5	26.2	35.7	1.3	3.6	4.9	13.7
AE GT 53 Japan	3.9	96.1	2.0	49.0	50.9	0.2	4.0	4.1	8.1
AE GT 60 China	13.3	86.7	5.4	35.2	40.6	0.4	2.6	3.0	7.4
AE GT 68 Ceylon	12.9	87.1	4.1	27.8	32.0	0.5	3.3	3.8	11.9
AE BT 7 India	9.7	90.3	1.6	14.7	16.2	0.1	1.2	1.3	8.3

[a] Data are expressed as mean ($n = 3$). MAE, microwave-assisted extraction; AE, Aquasolv extraction; GT, green tea; BT, black tea; dry wt, dry weight of tea.

unit. Approximately 3 g of tea samples were extracted in 30 mL of water at 100 °C for 30 min.

Microwave-Assisted Extraction. MAE was accomplished on an MLS-1200 Mega 40 instrument (MLS Ltd., Leutkirch, Germany) consisting of a degassing unit, a rotor block (HPR 1000/6M) holding six pressure-stable containers for samples, and an oven. Two different extraction programs for MAE were used: program 1 (MAE 1) (*32*) was carried out at 150 W for 200 s, ending with a ventilation period of 5 min, whereas program 2 (MAE 2) (*18*) consisted of several steps, that is, 700 W for 45 s, 0 W for 10 s, four times alternating steps consisting of 250 W for 3 s and 0 W for 10 s, and, finally, a ventilation period of 5 min.

In addition to the optimization of extraction method conditions, approximately 700 mg (708–738 mg) of samples were dissolved in 10 mL of different solvents [water, ethanol, and water/ethanol (50:50, v/v)] to evaluate the influence of the extraction solvent itself.

Aquasolv Extraction (33). Extraction was carried out using an Aquasolv apparatus (Berghof, Eningen, Germany) consisting of a boiler, a reactor, a cooling system, and autoclaves. Pressure and temperature were measured and recorded. The extraction process itself was performed under the following conditions: Approximately 830 mg of samples (810–857 mg) were placed in a closed autoclave and combined with 4 mL of extraction solvent. Three different solvent compositions [water, ethanol, and water/ethanol (50:50, v/v)] were evaluated as extraction solvents. Extraction was performed at a boiler temperature of 130 °C (1.8 bar) and a reactor temperature of 115 °C (1.5 bar). After 30 min, autoclaves were taken out of the reactor; samples were cooled, centrifuged, and analyzed.

Quantification of Polyphenols by Folin−Ciocalteu (*34*). A 7.5% (w/v) solution of sodium carbonate was prepared in water. An aliquot of 1 mL of sample was taken and combined with 5 mL of FC reagent (diluted 1:10 with distilled water). After 3–8 min, 4 mL of 7.5% (w/v) sodium carbonate solution was added. The mixture was incubated for 2 h at room temperature and measured at 740 nm employing a UV–visible spectrophotometer (UV-2000, Hitachi, Wiener Neudorf, Austria). A six-point calibration curve was established in the range of 10–200 ppm using gallic acid as standard ($R^2 = 0.9964$).

Reversed Phase High-Performance Liquid Chromatography Coupled to Electrospray Ionization Mass Spectrometry for Qualitative Analysis (RP-HPLC-ESI-MS) (System 1). To better understand the epimerization phenomenon, plant extracts were analyzed using an HPLC system coupled through an electrospray ionization interface to an LCQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The chromatographic separation system consisted of a Rheos 4000 micropump (Flux Instruments, Basel, Switzerland), an inline degasser (Brauer, Berlin, Germany), and a 10-port valve (Vici AG International, Schenkon, Switzerland) equipped with fused silica capillaries with defined volumes for injections (500 nL for μ-HPLC and 10 μL for analytical HPLC). In the μ-HPLC mode, a T-piece was connected to a restriction capillary (100 cm × 50 μm) to reduce the flow rate from 150 to 4 μL/min with a reasonable gradient delay. A 50 mm × 2 mm i.d., 5 μm, 120 Å, Prontosil RP-18 column (Bischoff Chromatography, Leonberg, Germany) was used as a stationary phase for HPLC separations and a *p*-methylstyrene-*co*-1,2-bis(*p*-vinylphenyl)ethane (*35*) based microbore column (6 cm × 200 μm, monolith) for μ-HPLC. The HPLC system was operated with a linear gradient using a mobile phase consisting of water/acetonitrile/formic acid (95:5:0.05, v/v/v; solvent A) and acetonitrile/formic acid (100:0.05, v/v; solvent B) at a constant flow rate 175 μL/min. Solvent flow was controlled by Janeiro II-SF (version 2.1, Flux Instruments, Basel, Switzerland). Zero time conditions were 95% A for 5 min, changing to 55% A from 5 to 25 min and to 0% A from 25 to 27 min. This state was retained for 5 min. At 34 min the mobile phase composition changed back to 95% A until the end of the run at 37 min. Separations were performed at room temperature.

MS investigations were performed in both positive and negative ionization modes using the manufacturer's software (XCalibur, vers. 1.3; Thermo Scientific). Mass spectra were recorded in the full-scan range from *m/z* 100 to 2000. Spray voltage was adjusted to 4.5 kV and capillary temperature set to 190 °C. Nitrogen (5.0; ≥99.999%, Messer Austria GmbH) was used as a sheath gas. MS tuning was accomplished using 0.1 mg/mL rutin standard.

Reversed Phase High-Performance Liquid Chromatography Coupled to Diode Array Detection and to Electrospray Ionization Mass Spectrometry for Quantitative Analysis (RP-HPLC-DAD-ESI-MS) (System 2). The HPLC-DAD-MS system (Shimadzu Corp.) consisted of a degassing unit (DGU-14A), two high-pressure gradient pumps (LC-10ADvp), an autosampler (SIL-10ADvp), a column oven (CTO-10Avp), a photodiode array detector (PDA; SPD-M10Avp), a single-quadrupole mass spectrometer (LCMS-2010), and a system controller (SCL-10Avp). Nitrogen for the MS was delivered by a nitrogen generator (N_2 LCMS, Claind, Lenno, Italy). The system control and data analysis were performed using the manufacturer's software packages (LCMS-Solution, version 3, and LCMS-Post run, version 3-H2). The stationary phase employed for quantification (studies) was a 50 mm × 2 mm i.d., 5 μm, 120 Å, Prontosil RP-18 column (Bischoff Chromatography). The composition of the mobile phase system was the same as described for system 1. Gradient elution was slightly modified. Zero time conditions at 0.5 mL/min were 98% A for 5 min. From 5 to 30 min, the composition changed from 98% A to 75% A and further to 55% A from 30 to 40 min, arriving at 0% A from 40 to 42 min. At 47 min starting conditions were re-established within 1 min. The end of the run was at 55 min. The injection volume was 10 μL, and the oven temperature was fixed to 30 °C. PDA operated in the wavelength range of 200–400 nm. Hyphenation to MS was performed by means of an ESI unit. Capillary voltage was adjusted to 4.5 kV, nebulizing gas (N_2) at 4.5 L/min, focusing potential at –200 V, entrance potential at 10 V, block heater temperature at 200 °C, and heated capillary temperature at 250 °C. Tuning was accomplished by using a mixture of different polyethylene glycols (PEGs) and raffinose. Full-scan data acquisition was performed by scanning from *m/z* 100 to 2000 in profile mode.

RESULTS AND DISCUSSION

Extraction of Analytes. The aim of extraction is, first of all, the solubilization of analytes into a liquid phase and, second,

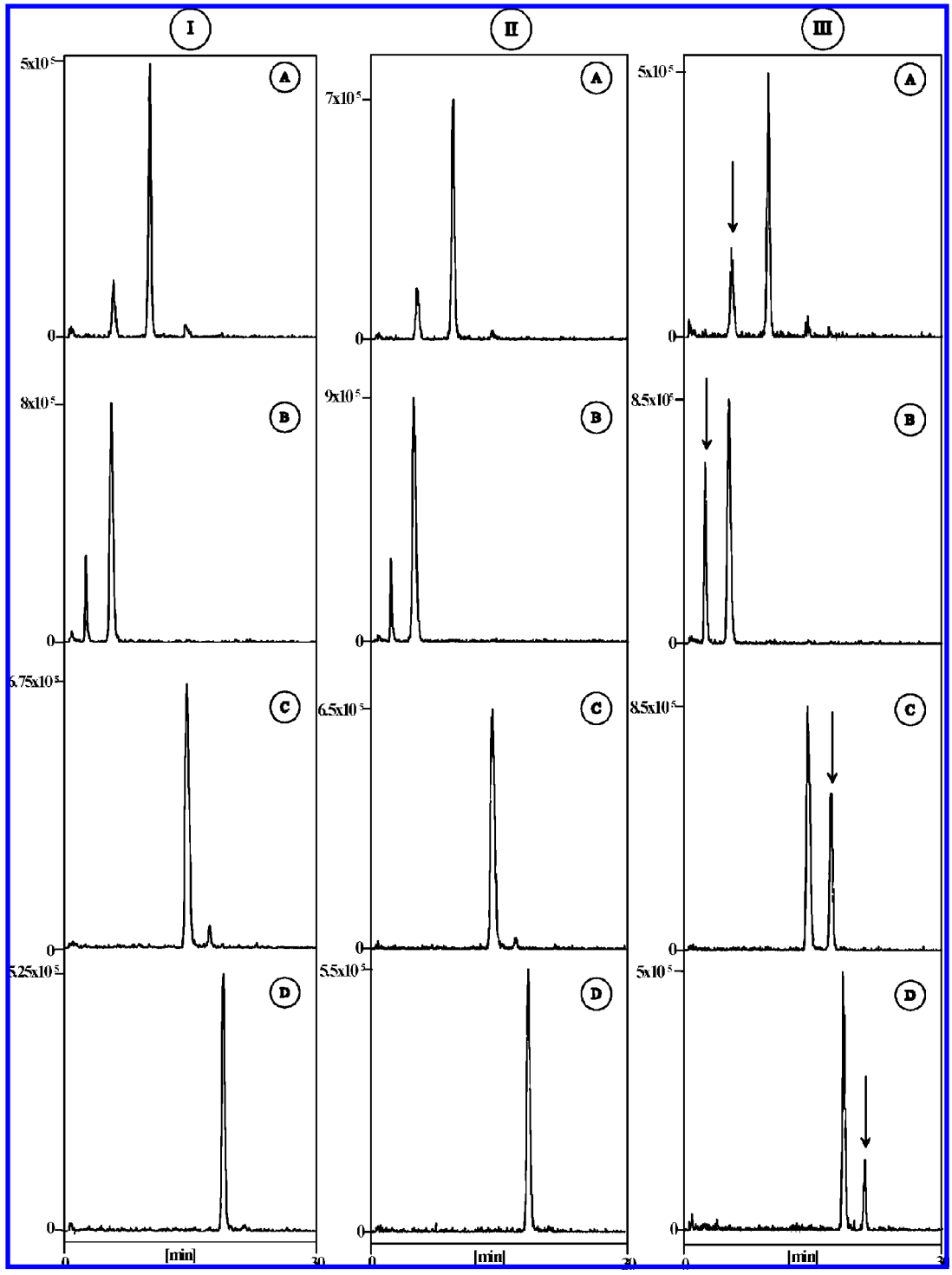

Figure 3. Influence of extraction method on epimerization of selected compounds: HPLC-MS (system 2) chromatograms from (**I**) TE 100% water, (**II**) MAE 100% water, and (**III**) Aquasolv extraction 100% water. (**A**) Catechin, epicatechin; (**B**) gallocatechin, epigallocatechin; (**C**) epigallocatechin gallate, gallocatechin gallate; (**D**) epicatechin gallate, catechin gallate.

the enabling of the determination and/or the structural elucidation of these analytes (*15*).

To obtain highest yields of solubilized tea ingredients such as catechin/epicatechin monomers and oligomers (**Figure 1**), different techniques have been used for evaluation. These include not only conventional, time-consuming, and laborious techniques such as reflux cooking (TE) but also more rapid and sophisticated ones, such as MAE (*18, 32*). Furthermore, Aquasolv extraction, a modified pressurized liquid extraction system, was considered for this evaluation (*33*). First experiments focused on the optimization of solvents by extracting the tea sample GT 48 India via MAE due to its high extraction efficiency in the shortest possible time. Six different solvents were evaluated concerning their extraction efficiency: **Figure 2A** clearly proves the high performance of organic phases such as alcohol or acetone mixed with water. As such, special emphasis was given to alcohol/water mixtures to be used for further extraction of all tea samples. These results were also confirmed by data obtained after quantification of selected compounds (**Figure 2B**) from the same GT sample.

For further comparison of extraction yields, mixtures of ethanol with purified water and tap water, respectively, were

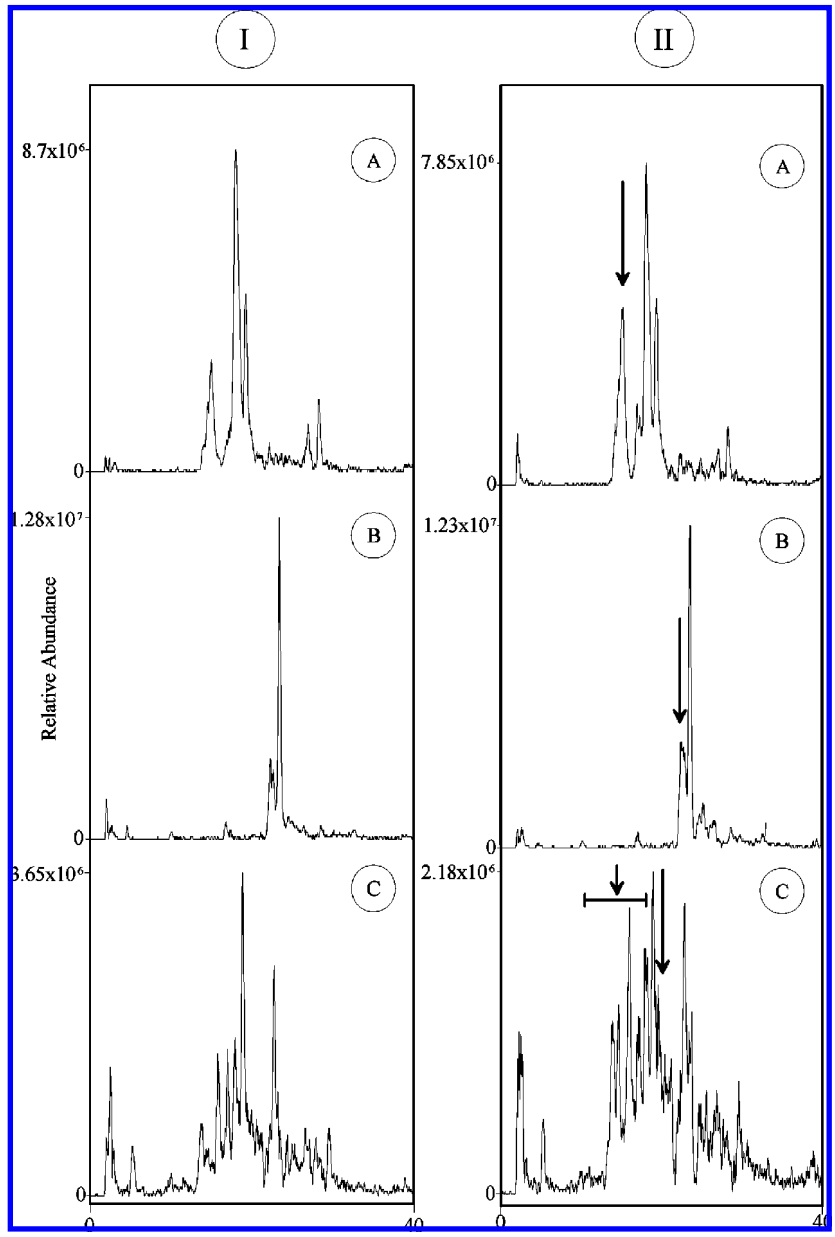

Figure 4. Influence of extraction method on epimerization of selected procyanidin oligomers: HPLC-MS (system 1) chromatograms from (**I**) MAE 100% water and (**II**) Aquasolv extraction 100% water. (**A**) Procyanidin P_2; (**B**) procyanidin gallate P_2G; (**C**) procyanidin P_3. The HPLC column was a Prontosil Eurobond column (125 × 4 mm, 5 μm).

used as solvents. The produced extracts were quantified by Folin−Ciocalteu (*34*) using gallic acid as standard. **Table 1** gives an overview about total tea polyphenols (TTPs) found in extracts after several techniques of extraction were employed. Generally, no significant difference between types of water could be found, as nearly all values were within the range of statistical variation. This was not the case for the gained yields of the employed techniques. MAE and traditional extraction rendered nearly comparable amounts of polyphenolic contents with an advantage of shorter extraction time for MAE. Aquasolv instead delivered significantly lower TTP values. In fact, MAE and traditional extraction rendered yields between 6 and 15% (using distilled water), whereas Aquasolv accounted for only 3−13.5%. Baptista et al. (*36*) found approximately 30% TTPs (content) in green tea, which is higher compared to the amount of TTPs recorded in the present work.

The variant procedures of MAE in programs 1 and 2 did not cause different results (data not shown), although intermediate cooling steps with 0 W power were introduced in program 2 to avoid overheating and, thus, loss of sample.

The variations in extraction performance between MAE and Aquasolv could be attributed to the fundamental differences between the techniques, for example, mechanisms of heating. Additionally, the elevated pressure (1.5 bar) during the Aquasolv process could have had some influence on overall yields of analytes.

Quantification of Selected Tea Ingredients by High-Performance Liquid Chromatography Hyphenated to UV Absorbance and Mass Spectrometric Detection (System 2). Quantification investigations were performed using mass spectrometric (MS) and UV (PDA) detection. UV detection was executed at two wavelengths, 254 and 280 nm, which correspond to the relative absorption maxima of selected flavonoids. Quantification was carried out via external calibration through regression analysis of signal intensities and corresponding standard concentrations. Correlation coefficients (R^2) using mass

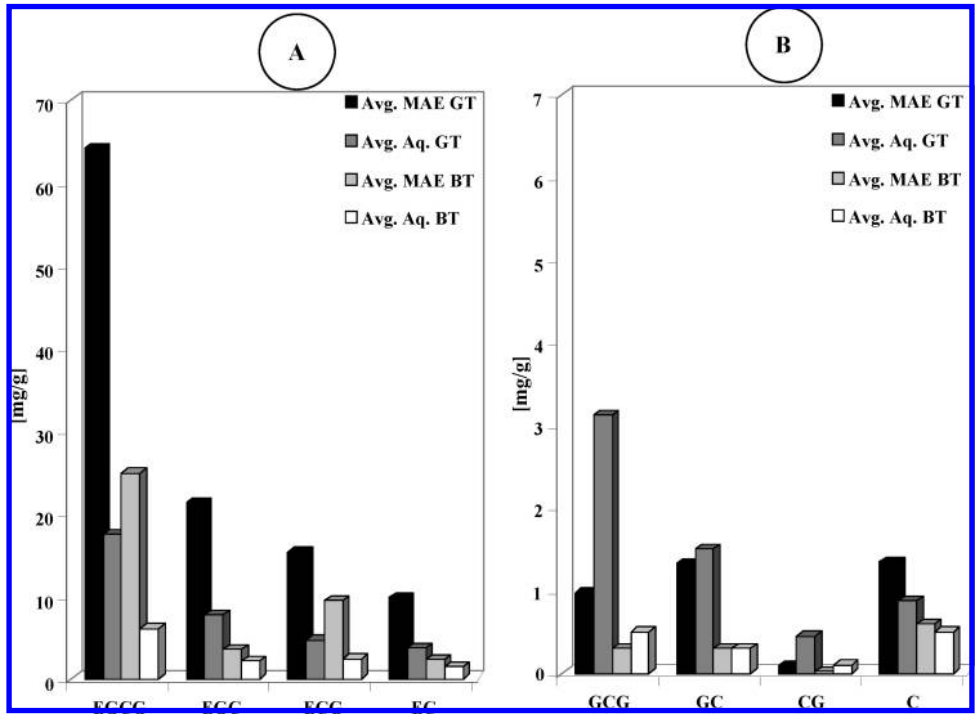

Figure 5. Average amounts of tea epicatechins (**A**) and their epimers (**B**) from different green and black tea samples (mg/g). Data are expressed as mean ± SD ($n = 3$). EGCG, epigallocatechin gallate; EGC, epigallocatechin; ECG, epicatechin gallate; EC, epicatechin; GCG, gallocatechin gallate; GC, gallocatechin; CG, catechin gallate; C, catechin.

spectrometric detection were 1 for nearly all standards, whereas photodiode array detection delivered R^2 values of 0.9940 for epicatechin gallate and catechin gallate (as the same calibration curve was used for both isomers) and of 1 for the other quantified substances. After the first HPLC injections of tea extracts, it became obvious that some compounds were present at high concentrations, whereas others were found in only relatively low amounts. Therefore, each sample extract was measured in the original (raw extract directly) concentration as well as in the diluted form.

Generally, both detection systems showed the same tendencies, but with higher yields for UV detection in comparison to corresponding data from MS detection. This is not in agreement with our previous findings concerning the separation and detection of wine flavonoids (*37*), for which we found significantly higher values for MS quantification than for UV detection. Nevertheless, the higher sensitivity and selectivity, especially for the interpretation of unidentified signals, prompted us to prefer mass spectrometry over UV detection.

The results of the quantitative investigation of all catechin derivatives (TCDs) from green tea and black tea samples are depicted in **Table 2**: total catechin derivatives (TCDs) correspond to the sum of total catechins (TCs), including catechin, gallocatechin, catechin gallate and gallocatechin gallate, and total epicatechins (TECs), comprising epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate. The proportion between TCs and TECs for MAE extracts was established between $^1/_{20}$ and $^1/_{49}$, that is, TCs accounted for 2.02–4.53% (w/w) of the total catechin derivatives (TCDs) and TECs for 95.73–98.07% (w/w). For Aquasolv extracts, the ratio between TCs and TECs was found in a range between $^1/_3$ and $^1/_{25}$. In this case TCs delivered between 3.88 and 26.58% (w/w) of TCDs and TECs between 73.42 and 96.12% (w/w).

In contrast to the described distribution of catechins and epicatechins, the contribution of TCDs to TTPs is strongly dependent on the type of tea besides the selected extraction method. In fact, for black tea samples the contents of TCDs in TTPs are only 32.46% (MAE) and 16.23% (Aquasolv). Within green tea samples TCDs contribute 79.38–90.13% to TTPs for MAE and 31.96–50.95% to TTPs for Aquasolv extraction, respectively. The same was noted when TCDs were calculated as a percentage of dry weight, but not when TTPs were calculated as a percentage of dry weight of tea. For TTPs no significant statistical differences between types of tea and methodologies of extractions were found (**Table 2**).

The differences between green and black tea samples are caused by fermentation during the production. Fermentation causes catechins to oxidize partially. This is followed by a complex condensation process forming theaflavins and thearubigins, which appear as the characteristic red and brown pigments of black tea. Chen et al. (*6*) found between 8 and 15% TCDs in green tea and 0.24−0.51% in black tea samples. Wang et al. (*19*) extracted approximately 20% TCDs in green tea and Mulder et al. (*13*), 10–25%. The results of the present study show approximately 11% TCDs in MAE green tea extracts and 3.5% in Aquasolv extracts.

The differences in quantified amounts of MAE and Aquasolv extraction will be discussed in more detail as they can possibly be ascribed to epimerization effects. **Figure 3** shows the LC-MS chromatograms of green tea extracts produced via traditional extraction (I), MAE (II), and Aquasolv extraction (III). It is evident that MAE and traditional extraction delivered approximately the same peak pattern. When the signals were quantified, a slight decrease of the epicatechin form and an increase in catechin epimer were observed in the case of the traditional extraction. By contrast, extracts produced with Aquasolv delivered strong changes in the epimer distribution. In fact, a significant increase was found for catechin (**Figure 3**, **III A**), gallocatechin (**Figure 3**, **III B**), gallocatechin gallate (**Figure 3**, **III C**), and catechin gallate (**Figure 3**, **III D**). This epimerization effect was strongest when plants were extracted with water, but it also took place during the extraction with

Table 3. Quantitative Determination of Individual Catechins in Tea Samples of Different Origins Extracted by Microwave-Assisted Extraction (MAE)[a]

	relative composition of individual catechin derivatives							
tea sample	EGCG	EGC	ECG	EC	GCG	GC	CG	C
MAE GT 48 India								
% to TCs (w/w)					17.5	39.6	2.5	39.78
% to TECs (w/w)	61.2	14.3	17.5	7.0				
% to TCDs (w/w)	58.6	13.7	16.7	6.7	0.7	1.7	0.1	1.70
% to TTPs (w/w)	51.3	12.0	14.6	5.9	0.7	1.5	0.1	1.49
% to dry weight of tea	6.7	1.6	1.9	0.8	0.1	0.2	0.0	0.2
MAE GT 53 Japan								
% to TCs (w/w)					39.9	22.1	2.2	36.4
% to TECs (w/w)	54.4	22.6	12.7	10.3				
% to TCDs (w/w)	53.3	22.1	12.4	10.1	0.8	0.5	0.1	0.8
% to TTPs (w/w)	48.1	19.9	11.2	9.1	0.7	0.4	0.04	0.7
% to dry weight of tea	5.6	2.3	1.3	1.1	0.1	0.1	0.0	0.1
MAE GT 60 China								
% to TCs (w/w)					41.6	34.1	5.3	17.3
% to TECs (w/w)	61.3	19.7	10.9	8.1				
% to TCDs (w/w)	60.1	19.3	10.7	8.0	0.8	0.7	0.1	0.3
% to TTPs (w/w)	47.7	15.3	8.5	6.3	0.7	0.5	0.1	0.3
% to dry weight of tea	6.5	2.1	1.2	0.9	0.1	0.1	0.0	0.0
MAE GT 68 Ceylon								
% to TCs (w/w)					20.4	37.0	2.0	40.0
% to TECs (w/w)	55.4	20.6	14.1	9.9				
% to TCDs (w/w)	52.9	19.7	13.4	9.5	0.9	1.7	0.1	1.8
% to TTPs (w/w)	42.6	15.9	10.8	7.6	0.7	1.3	0.1	1.5
% to dry weight of tea	6.8	2.5	1.7	1.2	0.1	0.2	0.0	0.2
MAE BT 7 India								
% to TCs (w/w)					28.5	21.8	1.9	48.1
% to TECs (w/w)	61.9	8.7	23.7	5.7				
% to TCDs (w/w)	60.1	8.5	23.0	5.5	0.8	0.6	0.1	1.4
% to TTPs (w/w)	19.5	2.7	7.5	1.8	0.3	0.2	0.0	0.5
% to dry weight of tea	2.5	0.3	0.9	0.2	0.03	0.03	0.0	0.1

[a] Data are expressed as mean ($n = 3$). EGCG, epigallocatechin gallate; EGC, epigallocatechin; ECG, epicatechin gallate; EC, epicatechin; GCG, gallocatechin gallate; GC, gallocatechin; CG, catechin gallate; C, catechin; TCs, tea catechins; TECs, tea epicatechins; TCDs, total catechin derivatives; TTPs, total tea polyphenols; MAE, microwave-assisted extraction; GT, green tea; BT, black tea.

mixtures of alcohol and water. It is interesting to note that this epimerization effect was also observed for oligomeric forms of epicatechin/catechin, among which especially dimeric and trimeric forms showed strong changes in the obtained pattern (**Figure 4**).

In agreement with the findings of Seto et al. (*38*), converted and unconverted catechins gave <100%. As shown in **Table 2**, the Aquasolv process actually delivered approximately one-third of TECs in comparison to MAE (see column TEC/dry wt in **Table 2**). Furthermore, TCs quantified with Aquasolv were not significantly higher, although epimerization was clearly observed in LC-MS chromatograms. As TTPs for both methods are comparable, it is still unclear what happens to TECs during the Aquasolv process. Most probably they are either oxidized or thermally degraded into products that are still able to be monitored by Folin−Ciocalteau's method.

To evaluate the occurrence of thermal degradation in the Aquasolv process, two standards, that is, quercetin and gallic acid, were treated under the same conditions. Generally, the antioxidant effect of flavonoids is enhanced when vicinal hydroxyl groups occur in the ring system and terminates with the oxidation of the flavonoid itself (*39*). This finally results in degradation products because of the high instability of the oxidized structure. Both quercetin and gallic acid possess such vicinal hydroxyl groups and were therefore chosen for the investigation of oxidation and degradation effects. After Aquasolv extraction and HPLC quantification, results clearly proved high stability of gallic acid, but they also demonstrated the significant loss of 20% of quercetin (dissolved in 50% ethanol/water). When the same experiment was repeated with MAE, this phenomenon was not observed. To clarify the contribution of solvents used, the same experiment was repeated with quercetin dissolved in 50% methanol/water (more polar than ethanol) and in 50% 1-propanol/water (less polar than ethanol). In all cases 10−20% of quercetin was lost.

Furthermore, differences in relative amounts can clearly be seen when single TEC and TC contributors, these are, epigallocatechin gallate, epigallocatechin, epicatechin gallate, and epicatechin for TECs as well as gallocatechin gallate, gallocatechin, catechin gallate, and catechin for TCs, were examined in more detail (**Figure 5**). All MAE extracts delivered clearly higher TECs in comparison to Aquasolv extracts. On the other hand, Aquasolv produced extracts with higher or equal amounts of TCs. This is a clear hint for epimerization effects occurring during the extraction process. Nevertheless, the degradation of targets is also evident, as average amounts of epigallocatechin gallate were approximately 65 mg/g for MAE and 18 mg/g for Aquasolv extracts (**Figure 5**). At the same time, quantified gallocatechin gallate amounts of 1 and 3.1 mg/g were obtained for both methods, respectively. To sum up, epimers (epigallocatechin gallate and gallocatechin gallate) result in 66 mg/g for MAE and in 21 mg/g for Aquasolv. As far as the amount in milligrams per gram of epigallocatechin gallate is concerned, the net difference between both methods is 47 mg, whereas its corresponding epimer gallocatechin gallate shows only an increase of 2.1 mg. It can be noted that if the epimerization phenomenon alone was involved in the case of Aquasolv extraction, then there should apparently not be any difference between total values of each pair of epimers. This implies the partial role played by thermal degradation in addition to the epimerization phenomenon as there is not 100% conversion. A similar tendency was found for epigallocatechin and gallocatechin, epicatechin gallate and catechin gallate, and epicatechin and catechin epimer combinations in both green and black tea samples.

Finally, **Table 3p**rovides an overview of quantified catechin derivates in different tea samples. Variations in content of target compounds and ultimately compositions of TCDs between different tea samples depend not only on the type and genetic properties of the tea itself but also on other factors such as soil, environmental conditions, season, climatic conditions, and manufacturing processes. In all five investigated samples epigallocatechin gallate was the major constituent, contributing approximately 54–62% to TECs and 52–60% to TCDs. As a part of TTPs, epigallocatechin gallate constituted 20–51% and finally contributed 2.5–6.8% to the dry weight of tea leaves (in the case of MAE). In green tea samples, respective concentrations of all TCDs were higher as compared to black tea samples. Epigallocatechin and epicatechin gallate delivered approximately one-third of the absolute amount. To compare, epigallocatechin gallate and epicatechin amount to one-sixth of the total. Therefore, a clear ranking in the order epigallocatechin gallate > epigallocatechin = epicatechin gallate > epicatechin can be defined. Among the epimers no grouping and tendencies were found.

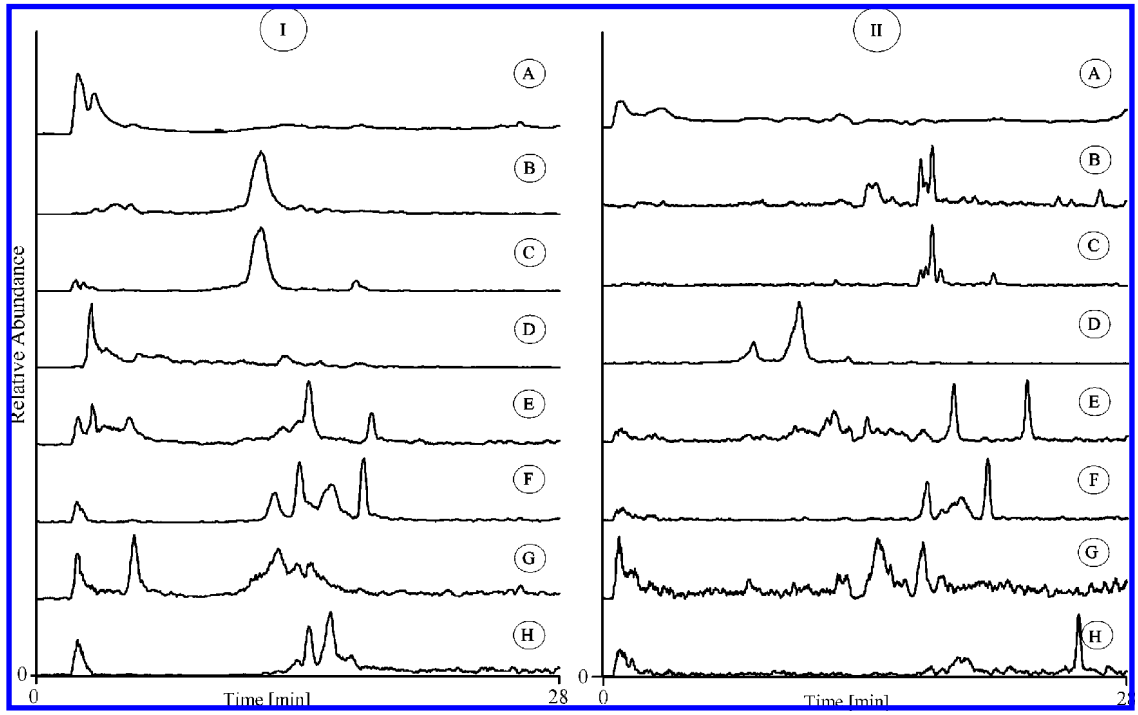

Figure 6. Tea extract separated by poly(*p*-methylstyrene-*co*-1,2-bis(*p*-vinylphenyl)ethane monolithic column (**I**) and by 50 mm × 2 mm Prontosil column (**II**). Mass spectrometric data: (**A**) TIC (total ion current), (**B**) *m*/*z* 286–288 (kaempferol), (**C**) *m*/*z* 448–450 (kaempferol-glycoside), (**D**) *m*/*z* 290–292 (catechin/epicatechin), (**E**) *m*/*z* 866–868 (P_3, procyanidin trimer), (**F**) *m*/*z* 1018–1020 (P_3G), (**G**) *m*/*z* 1154–1156 (P_4), (**H**) *m*/*z* 1306–1308 (P_4G). Separation conditions for (**I**): solvent A = 5% ACN in water + 0.05% HCOOH; solvent B = 100% ACN + 0.05% HCOOH; injection volume, 0.5 μL; gradient composition: [*t* (min), % B], [(0, 5), (5, 5), (25, 45), (27, 100), (32, 100), (34, 5), (37, 5)]; ionization source, ESI; spray voltage, 1.6 kV; heated capillary temperature, 200 °C.

Future Perspectives. Future investigations will focus mainly on the use of novel stationary phases for the separation of secondary plant metabolites. To save sample and mobile phase, miniaturization of columns receives primary attention. Promising results using a monolithic stationary phase such as *p*-methylstyrene-*co*-1,2-bis(*p*-vinylphenyl)ethane (MS/BVPE) (*35*) are shown in **Figure 6**.

Tea extract was separated using this monolith column, and chromatogram recordings were compared with data collected using a commercially packed 50 mm × 2 mm i.d., 5 μm, 120 Å, Prontosil RP-18 column (Bischoff Chromatography). A comparable separation efficiency between the two stationary phases was achieved, especially for procyanidin oligomers and corresponding gallates. An optimization of the stationary phase for small molecular weight analytes is still under consideration and in progress.

Conclusion. The present work is the contribution to the field of food chemistry with reference to the analysis of tea ingredients such as catechin and epicatechin derivatives. Various extraction techniques such as TE, MAE, and Aquasolv extraction were evaluated for their potential in obtaining high yields. Comparisons were performed on the basis of some parameters such as total tea polyphenols and quantification data for selected compounds such as catechin and epicatechin derivatives. As a result, MAE proved to be the method of choice, delivering highest extraction yields for quantified catechin derivatives within the shortest possible time. Nevertheless, especially the comparison with Aquasolv extraction gave rise to new findings. Differences between the two extraction methods were obtained due to thermal degradation and epimerization processes. Ultimately, these processes supported the epimerization of oligomeric catechin/epicatechin units, which was observed for the first time in this study.

ABBREVIATIONS USED

AE, Aquasolv extraction; BT, black tea; dry wt, dry weight of tea ; FC reagent, Folin−Ciocalteu reagent; GT, green tea; MAE, microwave-assisted extraction; PLE, pressurized liquid extraction; SE, Soxhlet extraction; SFE, supercritical fluid extraction; TCs, tea catechins; TCDs, total catechin derivatives; TE, traditional extraction ; TECs, tea epicatechins; TTPs, total tea polyphenols; UE, ultrasonic extraction.

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