DOI:10.1021/jf903614d

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Measurement of the Intracellular pH in Human Stomach Cells: A Novel Approach To Evaluate the Gastric Acid Secretory Potential of Coffee Beverages

Carola Weiss,[†] Malte Rubach,[†] Roman Lang,[‡] Elisabeth Seebach,[†] Simone Blumberg,[‡] Oliver Frank,[‡] Thomas Hofmann,*[,‡] and Veronika Somoza*[,†,§]

[†]German Research Center for Food Chemistry, Lichtenbergstrasse 4, D-85748 Garching, Germany, [‡]Chair of Food Chemistry and Molecular and Sensory Science, Technical University of Munich, Lise-Meitner-Strasse 34, D-84354 Freising, Germany, and [§]Research Platform Molecular Food Science, Althanstrasse 14 (UZA II), A-1090 Vienna, Austria

As the consumption of coffee beverages sometimes is reported to cause gastric irritation, for which an increased stomach acid secretion is one of the promoting factors, different processing technologies such as steam-treatment have been developed to reduce putative stomach irritating compounds. There is evidence-based data neither on the effect of detailed processing variations nor on individual coffee components affecting the proton secretory activity (PSA). This work aimed at developing a screening model suitable for investigating the effects of commercial coffee beverages and components thereof on human parietal cells. Human gastric cancer cells (HGT-1) were treated with reconstituted freeze-dried coffee beverages prepared from customary coffee products such as regular coffee (RC, $n = 4$), mild bean coffee (MBC, $n = 5$), stomach friendly coffee (SFC, $n = 4$), and SFC decaffeinated (SFCD, $n = 3$). PSA was analyzed by flow cytometry using the pH-sensitive dye SNARF-AM. Treatment of the cells with MBC did not result in a PSA different from RC treatment ($p \leq 0.07$), whereas cells treated with SFC ($p \leq 0.04$) or SFCD ($p \leq 0.03$) showed a significantly lower PSA than those treated with RC. Quantitative and principle component analysis of putative stomach irritating compounds revealed significantly reduced contents of $^{\beta}N$-alkanoyl-5-hydroxytryptamides, caffeine, *N*-methylpyridinium, and catechol in SFCD compared to RC. However, none of these compounds seem to act as the sole key bioactive reducing the PSA of SFCD, since their contents in MBC and SFC samples were not different from those in RC samples, although the PSA of these beverages was significantly lower than that of reconstituted freeze-dried RC beverage.

KEYWORDS: Gastric acid secretion; intracellular proton index; stomach irritation; HGT-1 cells; coffee

INTRODUCTION

Coffee ranges among the most popular beverages, next to spring water, and is highly appreciated for its pleasant aroma and its stimulating effect on the central nervous system. However, in some subjects, habitual coffee consumption causes heartburn or stomach irritation (*1*−*3*), which can be induced by, for example, an increased stomach acid secretion (*1*,*2*). Repeated hyperacidity of the stomach is well-accepted as one factor, among others, promoting stomach diseases, such as stomach ulcer or cancer over time (*4*). When compared to tap water or tea, the ingestion of 300 mL of coffee brew resulted in a significantly higher stomach acidity measured by a pH electrode positioned in the distal esophagus of healthy volunteers (*5*).

Literature reports suggest a contribution of the alkaloid caffeine to increased gastroesophageal reflux in healthy subjects. Studies on the effect of decaffeination on gastroesophageal reflux in patients with reflux disease revealed that, after removal of caffeine by supercritical carbon dioxide extraction, the coffee induced gastroesophageal reflux to a lower extent (*6*). However, the authors suggested that, in addition to caffeine, significant amounts of $^{\beta}N$-alkanoyl-5-hydroxytryptamides, a group of candidate stomach irritants with ulcerogenic effects on the gastric mucosa (*7*−*10*), were removed from the coffee bean by the decaffeination process, thus making the impact of caffeine still unclear.

Aimed at developing a "stomach-friendly" coffee, the steam treatment of raw coffee beans was introduced already 80 years ago (*11*−*13*). The main claim of the steam treatment technology, first invented in the 1930s by Lendrich et al. (*12*) and later improved by Darboven (*13*), is the partial removal of chlorogenic acids from raw coffee, as this compound is believed to decompose to give the candidate stomach-irritating hydroxybenzenes, such

+To whom correspondence should be addressed for the chemical analysis. Telephone: +49-8161/71-2902. Fax: +49-8161/71-2949. E-mail: thomas.hofmann@wzw.tum.de.

*To whom correspondence should be addressed for the cell assay. University of Vienna, Research Platform Molecular Food Science, Althanstrasse 14 (UZA II), A-1090 Vienna, Austria. Telephone: +43-1-4277-236-01. Fax: +43-1-4277-9-236. E-mail: veronika.somoza@univie.ac.at.

 Published on Web 01/13/2010

as, for example, catechol, 4-ethylcatechol, and pyrogallol (*14*), as well as caffeoyl quinides (*15*−*17*) upon roasting. Studies on the effect of regular and steam-treated coffee on gastric irritation and the intragastric pH-value in humans revealed that oral administration of a beverage prepared from steam-treated coffee induced significantly less mucosal irritation in the four healthy volunteers than the regular coffee brew, although the intragastric pH was not influenced (*11*).

Even though different technologies are applied to coffee beans to effectively reduce putative stomach irritating compounds, it is still not known which of these compounds stimulate the gastric acid secretion and if these stimulants are present in the raw bean or are formed upon roasting. Commercially, raw bean varieties which result in a less acidic coffee beverage are blended to produce a coffee product labeled as "mild bean coffee". These coffees are also widely believed to mildly affect the stomach function, although scientific evidence is lacking.

Another unanswered question is whether raw or roasted coffee beans contain compounds that reduce acid secretion or exhibit a stomach protective activity. In one of our previous studies, *N*-methylpyridinium ions, liberated from trigonelline upon roasting of coffee beans (*19*), was shown to have a chemopreventive activity in vitro and in vivo (*20*) and to downregulate the expression of the gastrin-receptor, which is involved in the molecular mechanism inducing the stomach acid secretion in parietal cells (*21*).

Therefore, the aim of the present study was to establish a cell culture model that permits the investigation of coffee beverages and their constituents for their effects on gastric acid secretion, as one of the main causes of stomach discomfort. The human parietal cell line HGT-1 was used as a model system, since this cell line expresses all functional and regulatory proteins that are related to gastric secretion (*22*, *23*). The most promising experimental approach to study the mechanism of stomach acid secretion so far has been to determine the depolarization of cell membranes after treatment of parietal cells with reconstituted freeze-dried coffee beverages by patch clamp techniques (*24*). However, this method is very useful for single cell-based assays but lacks the possibility of a high throughput screening using more than one cell per measurement. In this study, we developed a flow cytometric method for measuring the intracellular pH of human stomach cells (HGT-1 cells) using 1,5-carboxyseminaphtorhodafluor acetoxymethyl ester (SNARF-AM) as a suitable dye to measure intracellular pH in multicellular systems (*18*, *25*, *26*). The assay was validated by known modulators of gastric acid secretion such as histamine and omeprazole (*27*−*30*), respectively, and then applied to assess the stomach acid secretory activity of different coffee brews prepared from commercial samples of regular coffee, steam-treated coffee, decaffeinated coffee, and steam-treated and decaffeinated coffee. In addition, the candidate stomach irritants $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides (C5HT), caffeine, *N*-methylpyridinium, chlorogenic acid (CQA), chlorogenic acid lactones (CQL), and hydroxybenzenes (**Figure 1**) were quantitatively determined in these coffee brews. Finally, the effects of these coffees on the intracellular pH as an indicator of protons secreted into the stomach lumen were discussed in light of quantitative analytical data to give a first insight into their structure-specific activity.

MATERIAL AND METHODS

Chemicals. All chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany), solvents used were of HPLC quality, and water was Millipore quality. The internal standards d_3-pyrogallol (*31*), d_4-catechol (*31*), d_3-4-ethylcatechol (*31*), and d_3-*N*-methylpyridinium (*32*) were synthesized following literature protocols. Reference materials of

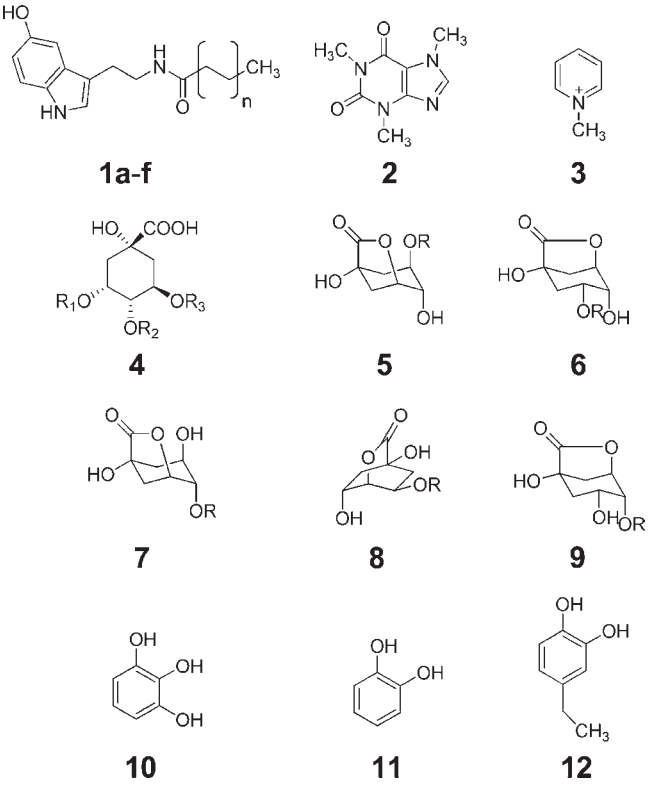

Figure 1. Chemical structures of candidate gastric acid modifiers in coffee beverages. $^{\beta}$*N*-Alkanoyl-5-hydroxytryptamides (**1**): $^{\beta}$*N*-stearoyl-5-hydroxytryptamide (**1a**; *n* = 16), $^{\beta}$*N*-arachinoyl-5-hydroxytryptamide (**1b**, *n* = 18), $^{\beta}$*N*-heneicosanoyl-5-hydroxytryptamide (**1c**, *n* = 19), $^{\beta}$*N*-behenoyl-5-hydroxytryptamide (**1d**, *n* = 20), $^{\beta}$*N*-tricosanoyl-5-hydroxytryptamide (**1e**, *n* = 21), and $^{\beta}$*N*-lignoceroyl-5-hydroxytryptamide (**1f**, *n* = 22); caffeine (**2**); *N*-methylpyridinium (**3**); chlorogenic acids (**4**) with 3-*O*-chlorogenic acid (R_1 = caffeic acid, $R_{2,3}$ = H), 5-*O*-chlorogenic acid (R_3 = caffeic acid, $R_{1,2}$ = H), 4-*O*-chlorogenic acid (R_2 = caffeic acid, $R_{1,3}$ = H); caffeoyl quinides (R = caffeic acid): 5-*O*-caffeoyl-*muco*-γ-quinide (**5**), 3-*O*-caffeoyl-γ-quinide (**6**), 4-*O*-caffeoyl-*muco*-γ-quinide (**7**), 5-*O*-caffeoyl-*epi*-δ-quinide (**8**), and 4-*O*-caffeoyl-γ-quinide **9**); hydroxybenzenes: pyrogallol (**10**), catechol (**11**), and 4-ethylcatechol (**12**).

3-*O*-caffeoyl-γ-quinide, 4-*O*-caffeoyl-γ-quinide, 5-*O*-caffeoyl-*epi*-δ-quinide, 4-*O*-caffeoyl-*muco*-γ-quinide, and 5-*O*-caffeoyl-*muco*-γ-quinide were prepared as published recently (*17*). Reference materials of $^{\beta}$*N*-stearoyl-, $^{\beta}$*N*-arachinoyl-, $^{\beta}$*N*-heneicosanoyl-, $^{\beta}$*N*-behenoyl-, $^{\beta}$*N*-tricosanoyl-, and $^{\beta}$*N*-lignoceroyl-5-hydroxytryptamides were synthesized as reported recently (*33*).

Sample Preparation. Sixteen commercial coffee samples were purchased from local grocery stores and divided into the following four groups according to the product declaration: regular coffee (RC, *n* = 4), mild bean coffee (MBC, *n* = 5), stomach-friendly coffee (SFC, *n* = 4), and stomach-friendly coffee decaffeinated (SFCD, *n* = 3). Coffee beverages were prepared following a standard recipe which is typically used for household preparations. From a batch of ground roast coffee powder, 54 g of homogeneous material were weighed into a cellulose filter (Melitta Gold, Nr. 4, Aldi, Germany) and then percolated with portions (100 mL each) of hot tap water (95 °C) until 1000 mL of the coffee beverage were obtained. After cooling to room temperature in an ice-bath, the coffee brew was freeze-dried for 48 h (0.77 mbar), and the yield of the extract was determined by gravimetry. Total yields obtained after freeze-drying of the commercial samples did not vary significantly and were in the range of 28 ± 2%.

Cell Culture. The human parietal carcinoma cell line HGT-1 was obtained from Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes France). The cells were cultured under standard tissue culture conditions at 37 °C and 5% CO_2. Dulbeccos Modified Eagle Medium (DMEM, PAA, Coelbe, Germany) with glucose (4%, PAA, Coelbe, Germany) was used as culture medium and supplemented with 20% fetal

calf serum (PAA, Coelbe, Germany), 2% L-glutamine (PAA, Coelbe, Germany), 2% penicillin streptomycin (PAA, Coelbe, Germany), and 2% 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffer (PAA, Coelbe, Germany). Cells were seeded in a density of 500 000 cells in a T75 flask, and the cell growth was controlled by the transepithelial electrical resistance. Cells were cultivated with FCS for a period of 4−5 days, since at this time they showed a confluence of 90−100%. Synchronization of the cells was performed for 24 h in media without FCS. According to the literature, HGT-1 cells are well differentiated for experiments at the confluent state (*22*). For the flow cytometry assay, the cells were washed once with phosphate-buffered saline (PBS; PAA, Coelbe, Germany) and were harvested with a cell scraper after accutase (PAA, Coelbe, Germany) treatment. The suspended cells were counted using a hemocytometer (Neubauer, VWR, Germany), and the viability was assessed by trypan blue (PAA, Coelbe, Germany) staining and using an automated cell counter (Schärfe System, Reutlingen, Germany). Cells were finally adjusted to a concentration of 1 million viable cells per milliliter of PBS.

Secretory Activity Measurement by Flow Cytometry. An aliquot (2 mL) of the cell suspension, corresponding to 2,000,000 cells, was used for each biological independent experiment and was incubated with 2.5 mg of coffee lyophilizate per mL of PBS at 37 °C for 10 min. Afterward, the fluorescent pH-sensitive dye 1,5-carboxy-seminaphtorhodafluor acetoxymethyl ester (SNARF-AM, Invitrogen, Karlsruhe, Germany) was added in a concentration of 3 μmol/L and the incubation was continued on ice for another 30 min. Finally, the cells were centrifuged at 300*g* for 5 min and resuspended in ice-cold PBS prior to the flow cytometric measurement. A calibration curve was generated for each experiment by staining the cells in potassium buffers of varying pH values ranging from 6.4 to 8.2 and adding 2 μmol/L of the ionophor nigericin (PAA, Coelbe, Germany) to equilibrate intracellular pH (pH_i) and extracellular pH (pH_{ex}). The composition of the calibration K^+-clamp buffers was as follows: 20 mmol/L NaCl, 110 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 18 mmol/L D-glucose, and 20 mmol/L HEPES that was adjusted to different pH values (6.4−8.2) by titration with NaOH.

The flow cytometer (FACSCalibur, Becton & Dickinson, Heidelberg, Germany) was used to excite the dye with 500 mW of the 488 nm argon laser line. For the calibration curve and for the sample-treated cells, two fluorescent bands (FL-1 = 550−590 nm, FL-2 ≥ 600 nm) were collected as an integral signal, and the ratio of these (FL-2/FL-3) was calculated with an analog function board. The ratio channel number is linearly related to the actual ratio FL-2/FL-3, with the ratio of 1 being in channel 256. The filter combination to achieve these fluorescent bands was a 550 nm blocking filter, a 590 nm dichroic filter, and a 610 nm long-pass filter. The pH_i calibration was fit to a linear regression, and the fitted parameters were used to generate an equation which converted the ratio channel number (FL-2/FL-3) from the medians of 10,000 cell counts to pH_i. The concentration of intracellular H^+ was calculated from the pH_i and is expressed in nmol/L for the validation experiments. For all other experiments, the intracellular proton index (IPX) was calculated by $\log_2$ transformation of the intracellular proton concentration ratio between treated cells and control cells. Histamine (1 mmol/L), as physiological stimulant, and omeprazol (1 mmol/L) (both Sigma-Aldrich, Munich, Germany), as a well-known inhibitor of stomach acid secretion, were used as control compounds. In initial experiments with reconstituted freeze-dried coffee-treated cells, time course and dose response studies were performed to identify the concentration and treatment time resulting in the most pronounced effect on the IPX. Finally, HGT-1 cells were treated with 2.5 g/mL of each of the reconstituted freeze-dried beverages prepared from the commercial coffee samples for 10 min.

High-Performance Liquid Chromatography (HPLC). The HPLC-DAD system consisted of a L-7100 pump (Merck/Hitachi), a L-7612 degasser, a L-7200 autosampler, and a L-7450 detector monitoring the effluent at a wavelength from 230 to 400 nm. For the analysis of caffeine, the wavelength of 272 nm was extracted from the spectra, whereas chlorogenic acids and caffeoyl quinides were analyzed at 324 nm. The hardware was connected to the computer via an interface (Merck/Hitachi D-7000), and the software used for data acquisition and processing was the HPLC system manager 4.1.

Quantitative Analysis of Caffeine, Chlorogenic Acids, and Caffeoyl Quinides, Respectively. For the quantitative analysis of caffeine and the individual chlorogenic acids, namely 3-*O*-, 4-*O*-, and 5-*O*-caffeoyl quinic acid, freshly prepared coffee brew was diluted with water (1 + 9, v:v) and, after membrane filtration (0.45 μm), an aliquot (10 μL) was injected into the HPLC-DAD-system. For quantitation of the caffeoylquinides, namely 3-*O*-caffeoyl-γ-quinide, 4-*O*-caffeoyl-γ-quinide, 5-*O*-caffeoyl-*epi*-δ-quinide, 4-*O*-caffeoyl-*muco*-γ-quinide, and 5-*O*-caffeoyl-*muco*-γ-quinide (*17*), freshly prepared coffee brew was diluted with water (1:1, v:v) and, after membrane filtration (0.45 μm), an aliquot (20 μL) was injected into the HPLC-DAD system. Chromatography was performed on a 4.6 mm × 250 mm, 5 μm, Luna phenyl-hexyl column (Phenomenex, Aschaffenburg, Germany) operated at a flow rate of 0.8 mL/min. Eluent A was an aqueous ammonium formate solution (0.25 mmol/L), adjusted to pH 3.5 with concentrated formic acid, and eluent B was methanol. Eluent B was increased from 22 to 25% within 25 min and then to 100% within 5 min, followed by isocratic elution for 5 min. Quantitation was performed using a 5-point external calibration in the concentration range from 5 to 500 μmol/L. Caffeine, caffeoylquinic acids, and caffeoyl quinides were quantified using caffeine and 5-caffeoylquinic acid, respectively, as external standards.

Quantitative Analysis of $^{\beta}$*N*-Alkanoyl-5-hydroxytryptamides. $^{\beta}$*N*-Alkanoyl-5-hydroxytryptamides, namely $^{\beta}$*N*-stearoyl-, $^{\beta}$*N*-arachinoyl-, $^{\beta}$*N*-heneicosanoyl-, $^{\beta}$*N*-behenoyl-, $^{\beta}$*N*-tricosanoyl-, and $^{\beta}$*N*-lignoceroyl 5-hydroxytryptamides, were quantitatively determined by means of the stable isotope dilution analyzes developed recently (*33*). Briefly, an aliquot of the coffee brew was spiked with the stable isotope labeled internal standards, mixed and diluted with methanol. After membrane filtration (0.45 μm), the samples were injected into the LC-MS/MS system (5 μL).

Quantitative Analysis of Hydroxybenzenes. The hydroxybenzenes pyrogallol, catechol, and 4-ethylcatechol were quantitatively determined by means of stable isotope dilution analyses developed previously (*31*). The coffee brew sample (25 mL) was placed into a 80 mL centrifugation tube with a screw cap (Schott AG, Mainz, Germany), defined amounts of the internal standards d_3-pyrogallol, d_4-catechol, and d_3-4-ethylcatechol were added, and, after closing the tube, the sample was equilibrated for 20 min at room temperature under an atmosphere of nitrogen while stirring. The solution was extracted with diethyl ether (25 mL) and centrifuged (10 min, 3000 rpm), the organic supernatant was separated, and the solvent was removed in vacuum. The residue was taken up in methanol/water (1:1, v:v; 1 mL) and membrane filtered (0.45 μm), and aliquots (5 μL) were analyzed by means of HPLC-MS/MS.

Quantitative Analysis of *N*-Methylpyridinium. Quantitation of *N*-methylpyridinium ions was performed by means of a stable isotope dilution analysis following a literature protocol (*20*, *32*) with some minor modifications. An aliquot of the freshly prepared coffee beverage (1 mL) was spiked with an aqueous solution of d_3-*N*-methylpyridinium iodide (1.5 μmol/mL; 100 μL). After mixing and equilibration for 10 min, the solution was applied onto a CBA cation exchange SPE-cartridge (1 g, 5 mL; Varian, Darmstadt, Germany), which had been conditioned with methanol, followed by water (5 mL each). After application of the sample, the cartridge was rinsed with water and methanol (5 mL each), followed by a solution of 20% aqueous formic acid (5 mL). The acidic effluent was diluted with water (1:1, v:v), and an aliquot (5 μL) was analyzed by means of HPLC-MS/MS. Chromatography was performed isocratically with a mixture (1:1, v:v) of methanol and aqueous ammonium acetate (50 mmol/L) on a 50 mm × 2 mm, 5 μm, GROM-Sil80 SCX column (Alltech Grom GmbH, Rottenburg-Hailfingen, Germany). Using the multiple reaction monitoring (MRM) mode, the transitions from the parent ion $[M]^+$ to the fragments after collision-induced dissociation were recorded as follows: *N*-methylpyridinium ion: m/z 94 → m/z 79, m/z 94 → m/z 78, and m/z 94 → m/z 52; d_3-*N*-methylpyridinium: m/z 97 → m/z 79, m/z 97 → m/z 78, and m/z 97 → m/z 52. Calibration of the internal standard was performed by analyzing mixtures of analyte and internal standard in molar ratios from 1:10 to 10:1. A regression line was calculated from a graph prepared from area ratios (analyte/internal standard) vs concentration ratios (analyte/internal standard). The equation was $y = 1.1108x + 0.002$ ($R^2 = 0.9992$).

High Performance Liquid Chromatography/Mass Spectrometry (HPLC-MS/MS). The Agilent 1200 Series HPLC-system (Agilent, Waldbronn, Germany) consisting of a pump, a degasser, and an autosampler was connected to a 3200 API triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Darmstadt) equipped with an electrospray ionization (ESI) source. The software used for instrumentation control and data collection was analyst v1.4.1 (Sciex, Toronto, Canada).

Table 1. Concentrations of Nitrogen-Containing Coffee Constituents[a] in Brews Prepared from the Product Categories Regular Coffee (RC), Mild-Bean Coffee (MBC), Stomach-Friendly Coffee (SFC), or Stomach-Friendly Decaffeinated Coffee (SFCD)

		concentration (μg/L) of							concentration (mg/L) of	
sample		**1a**	**1b**	**1c**	**1d**	**1e**	**1f**	$\sum$**1a−f**	**2**	**3**
RC	A	3.8	114.3	1.8	303.9	5.4	46.1	475.3	713.8	19.9
	B	2.4	76.3	1.5	168.3	4.3	22.6	275.4	598.4	20.8
	C	3.1	80.6	2.5	182.7	3.3	25.3	297.5	618.6	19.3
	D	1.5	42.9	1.3	87.2	3.1	13.7	149.7	620.5	38.8
								299.5 ± 134.1^{a}	637.8 ± 51.6^{a}	24.7 ± 9.4^{a}
MBC	E	4.5	87.9	3.1	171.8	3.9	24.5	295.7	752.0	25.3
	F	3.4	94.8	1.7	251.6	6.1	47.1	404.7	608.4	20.9
	G	3.9	81.3	1.5	162.4	4.9	25.2	279.2	735.2	22.9
	H	3.3	77.8	1.7	162.9	3.5	23.4	272.6	688.0	29.3
	I	5.2	133.5	1.9	300.5	5.8	42.0	488.9	656.9	24.9
								348.2 ± 95.2^{a}	688.1 ± 58.4^{a}	24.7 ± 3.1^{a}
SFC	J	2.8	100.4	2.6	251.7	5.1	38.0	400.6	729.5	17.5
	K	3.6	87.8	2.2	185.0	4.7	24.6	307.9	759.5	21.8
	L	2.7	97.8	1.6	199.4	5.1	31.8	338.4	815.5	21.8
	M	1.8	85.9	1.7	182.5	3.3	23.6	298.8	638.1	21.2
								336.2 ± 46.0^{a}	735.7 ± 74.2^{a}	20.6 ± 2.1^{a}
SFCD	N	1.1	16.9	0.8	30.1	1.7	11.9	62.5	35.1	12.9
	O	1.2	17.2	0.7	32.3	1.8	11.8	65.0	18.8	14.8
	P	0.9	15.6	0.5	31.1	1.7	11.4	61.2	17.3	18.9
								62.9 ± 1.9^{b}	23.7 ± 9.9^{b}	15.5 ± 3.1^{b}

[a] The nitrogen-containing compounds are $^{\beta}$*N*-stearoyl-5-hydroxytryptamide (**1a**), $^{\beta}$*N*-arachinoyl-5-hydroxytryptamide (**1b**), $^{\beta}$*N*-heneicosanoyl-5-hydroxytryptamide (**1c**), $^{\beta}$*N*-behenoyl-5-hydroxytryptamide (**1d**), $^{\beta}$*N*-tricosanoyl-5-hydroxytryptamide (**1e**), $^{\beta}$*N*-lignoceroyl-5-hydroxytryptamide (**1f**), caffeine (**2**), and *N*-methylpyridinium (**3**). Different index letters in each row indicate significant differences between the respective product categories ($p < 0.05$).

The MS-parameter settings for the analysis of $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides (*33*) and hydroxybenzenes (*32*) were the same as those published recently. For the analysis of *N*-methylpyridinium (NMP), the ESI source was operated in positive mode, nitrogen was used as the nebulizer gas (65 psi) and curtain gas (25 psi), respectively, the heater gas was set at 60 psi, and the temperature was 350 °C. The declustering potential (DP) was 50, the cell exit potential (CXP) was 15, and the collision energy (CE) was 33. The dwell time for each mass transition was 50 ms. The quadrupoles operated at unit mass resolution, and the ion spray voltage was 5500 V.

Statistical Analysis. Statistics were performed with Excel 2003 (Microsoft, Munich, Germany) and SigmaStat (Systat Software GmbH, Erkrath, Germany), respectively. Single comparisons between treated and control cells were done with the two-tailed Student's *t*-test for equal variances unless otherwise stated. For time course and dose−response analysis, a one-way ANOVA was performed with a Holm−Sidak posthoc test. The number of independent biological replicates for each treatment was at least $n = 3$. Data are presented in mean values ± standard error (SE). Analytical data on coffee beverage constituents is given as the mean of duplicates with relative standard deviation < 10%, and mean values of individual categories were analyzed by a one-way ANOVA with Holm−Sidak posthoc test.

Principal Component Analysis (PCA). PCA was computed by using the software Systat version 12.0 (Systat Software GmbH, Erkrath, Germany). PCA was performed to identify associations between the differentially labeled commercial coffees. As parameter set for extraction of the loading factors for a single coffee beverage, we used the content of each compound quantified in the corresponding beverage. For those compounds with equal chemical structure but different substitutions, such as $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides and caffeoyl quinides, we used the sum value. The settings for PCA were used as follows: minimum eigenvalue = 1, factors = 4 (according to the four different types of coffee beverages), rotation = varimax. We performed various PCAs in order to determine the influence of single compounds on the association between single coffee beverages. First, a PCA including the quantified contents of all compounds and, second, a PCA was applied to those compounds that showed significant differences among the tested beverages, such as catechol, *N*-methylpyridinium, caffeine, and $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides (**Table 1**).

RESULTS AND DISCUSSION

In order to evaluate the gastric acid secretory potential of coffee brews, a screening model using the human stomach cell line HGT-1 had to be developed. Previously published studies using HGT-1 cells examined the constitution of HGT-1 cells and their ability to express those proteins which are involved in the mechanism of proton secretion. To the best of our knowledge, the here presented work describes the effect of food extracts on proton secretion for the first time. Carmosino et al. 2000 (*22*) characterized the mechanisms of proton secretion in HGT-1 cells using a pH-sensitive fluorescent dye but did not address the question whether food components affect the transport systems of acid secretion. From a technical point of view, the cellular uptake of radiolabeled aminopyrine by stimulated parietal cells may serve as an indicator of proton secretion (*34*). Also, Fiebich et al. (*24*) applied a patch clamp technique to analyze potassium and chloride ion fluxes as indicators of proton secretion. Compared to the here presented method by which the intracellular pH is measured as a direct consequence of proton secretion, the methods published by Berglindh et al. (*34*) and Fiebich et al. (*24*) analyze only indirect markers of proton fluxes.

The here presented assay was applied to commercial coffee samples of different product categories to find out whether the differences in processing technologies indicated by the product label result in specific effects on the intracellular proton index (IPX) as a measure of proton secretion.

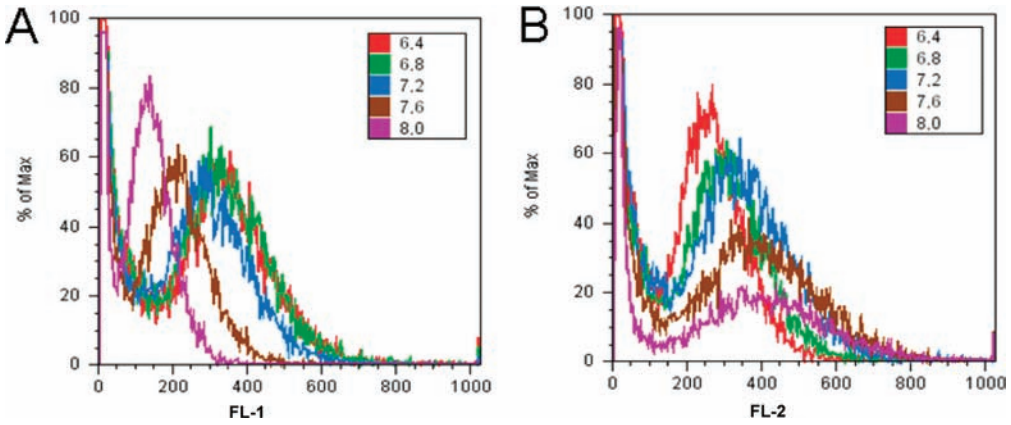

Figure 2. FL-1 (**A**) and FL-2 (**B**) fluorescent intensity of HGT-1 cells after treatment with potassium buffer solutions adjusted to pH values ranging from 6.8 to 8.0, and the fluorescent dye SNARF-AM (3 μmol/L).

Quantitative data of putative stomach irritating coffee compounds and those of which the concentrations are known to differ among commercially applied processing technologies were analyzed and discussed in light of the functional IPX data.

Assay Calibration. The assay for assessment of secreted protons by HGT-1 cells is based on intracellular pH-measurement using the fluorescent dye SNARF-AM. When cultivated cells are treated with potassium calibration buffers of varying pH values ranging from 5.5 to 8.4 in the presence of the ionophor nigericin, the intracellular pH (pH_i) and the extracellular pH (pH_{ex}) equilibrate. This allows the determination of pH_i by analyzing the pH-characteristic fluorescence of SNARF-AM (*18*, *25*, *26*). In this study, treatment of HGT-1 cells with potassium calibration buffers adjusted to various pH values between 6.8 and 8.2 accordingly resulted in characteristic changes of SNARF-AM fluorescence. Increasing intracellular pH values shifted the intensity of FL-1 to lower counts, whereas the intensity of FL-2 was shifted to higher counts (**Figure 2**). Fluorescent FL-2/FL-3 channel ratios and pH values of the calibration buffers ranging from pH 6.8 to 8.2 were fit to a linear regression with a correlation coefficient of $R^2 = 0.9905$ and $y = 0.5163x \pm 5.026$, and the values were used to calculate pH_i and the intracellular concentration of protons in sample-treated cells. In the next set of experiments, we aimed at testing whether histamine, a well investigated stimulant of stomach acid secretion (*30*), as well as omeprazol, a potent inhibitor (*29*), show the expected effects and, thus, prove the applicability of the assay. Treatment of the HGT-1 cells with histamine resulted in an 8% (two-tailed *t*-test; $p \leq 0.001$) decrease in intracellular proton concentration, indicating an increased proton efflux compared to the case of nontreated control cells. In omeprazol treated cells, in contrast, the concentration of protons increased by 22% (two-tailed *t*-test; $p \leq 0.001$), which indicates a reduced proton efflux compared to the case of controls (**Figure 3**).

Dose and Time Dependent Effects of Regular Coffee on IPX. Assuming that activation of proton secretion is dose dependent and takes place in a short or midterm response in HGT-1 cells, dose and time-course experiments were performed. For data calculation, a $\log_2$ transformation of the intracellular proton concentration ratio between treated cells and control cells was performed and expressed as intracellular proton index (IPX). In general, variance stabilization is one of the primary reasons that raw data are $\log_2$-transformed before further analysis, in particular when larger intensities tend to have larger variations when repeatedly measured. This violation of a constant variance across the measurement range imposes a serious challenge when applying parametric tests, such as the Student's *t*-test, or analysis of variance (ANOVA) (*35*).

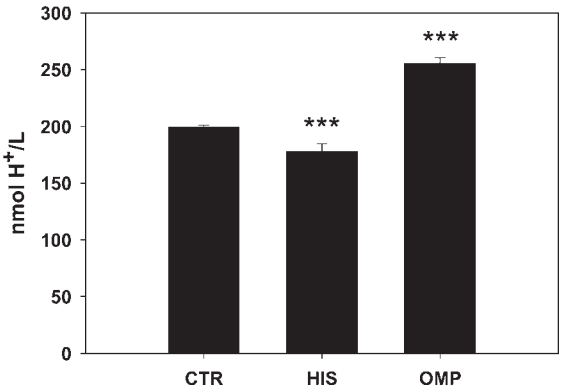

Figure 3. Intracellular proton concentration of HGT-1 cells treated with histamine (HIS, 1 mmol/L) or omeprazole (OMP, 1 mmol/L) compared to nontreated control cells (bars show mean values ± SE; Student's one-tailed *t*-test: *** = $p < 0.001$; $n = 4-6$).

Although $\log_2$-transformation is mainly applied to microarray data (*36*), the data presented here were $\log_2$-transformed, as we expected a broad range of effect magnitude for the very heterogeneous group of commercial coffee samples, which were not standardized for, i.e., coffee variety or processing techniques. Thus, we could not exclude the hypothesis of "larger intensities" having larger variations and, therefore, defined the IPX based on the hypothesis that, in this assay, the intracellular concentration of H^+ decreases due to proton secretion. The higher the IPX, the more protons remain in the cell, indicating a lower secretory activity, whereas lower IPX values indicate a higher proton secretion.

For the dose and time-course experiments, the IPX was measured after 5, 10, 20, and 30 min of treating the cells with aqueous solutions of reconstituted freeze-dried regular coffee in concentrations ranging from 0.5 up to 5.0 mg/mL (**Figures 4**A and **4**B). The lowest IPX values, indicating the highest proton efflux, were demonstrated for cells treated with 2.5 mg/mL of reconstituted freeze-dried coffee beverage for 10 min (both one-way ANOVA; $p \leq 0.05$). The decrease of the IPX to -0.10 ± 0.01 after treatment with a concentration of 2.5 mg/mL was tested to be significantly lower compared to the case for other concentrations by Holm−Sidak posthoc analysis ($p \leq 0.05$, **Figure 4**A). A time-dependent minimum of -0.18 ± 0.02 in IPX after treatment with the reconstituted freeze-dried regular coffee beverage (2.5 mg/mL) was demonstrated after 10 min of exposure (**Figure 4**B). The IPX at this time point was significantly different (one-way ANOVA, Holm−Sidak posthoc test: $p \leq 0.05$) from those for

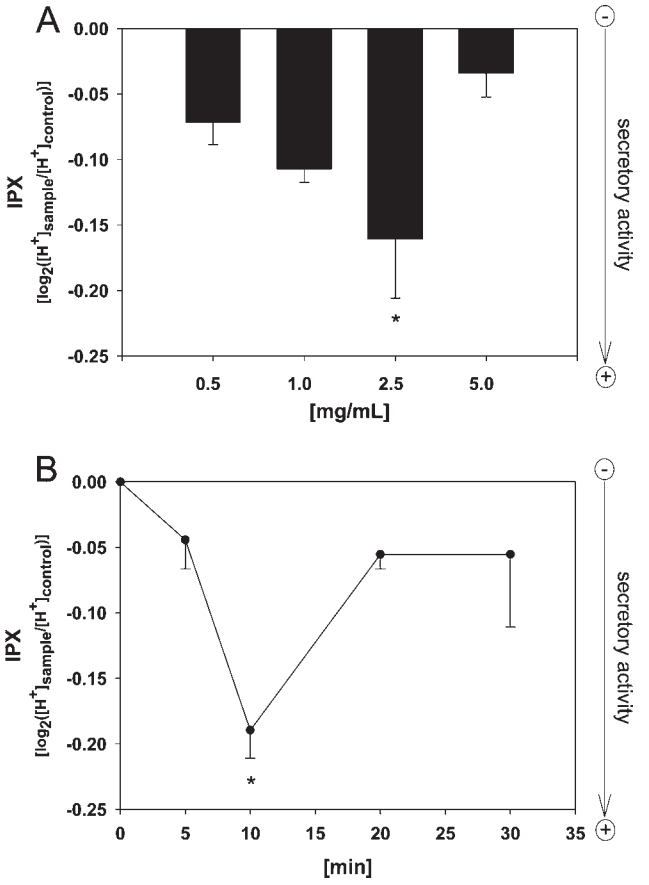

Figure 4. Time and dose dependent stimulation of proton secretion by reconstituted freeze-dried regular coffee in HGT-1 cells. (**A**) intracellular proton index (IPX) in HGT-1 cells after treatment with 0.5, 1.0, 2.5, or 5.0 mg/mL of a lyophilizate prepared from regular coffee (54 g/L) for 10 min; (**B**) intracellular proton index (IPX) in HGT-1 cells after treatment of 2.5 mg/mL of a lyophilizate prepared from regular coffee (55 g/L) for 0, 5, 10, 15, 20, 25, or 30 min (bars show mean values ± SE; one-way ANOVA, Holm−Sidak posthoc test: * = $p < 0.05$; $n = 3-10$).

the other treatment times tested. The precision of this assay, expressed as the coefficient of variance (CV) obtained by ten replicate treatments of cells of one passage with the same sample, was below 2% (data not shown). Hereby, the HGT-1 model system was optimized for the measurement of proton secretory activities induced by reconstituted freeze-dried coffee beverages.

Determination of the Proton Secretory Activity of Coffee Samples. A total of 16 commercial coffee samples was classified according to labels into the group of regular coffee (RC), mild bean coffee (MBC), stomach friendly coffee (SFC), and stomach friendly coffee decaffeinated (SFCD), and their proton secretory activities were determined by means of the IPX measurement (**Figure 5**). Whereas MBCs are commonly coffee blends exhibiting a somewhat lower acidity when compared to RC, stomach friendly coffees are steam-treated prior to roasting, and decaffeinated stomach friendly coffees are extracted with carbon dioxide or organic solvents followed by steam treatment and roasting.

Treatment of the HGT-1 cells with reconstituted freeze-dried beverages from RC in a concentration of 2.5 mg/mL for 10 min resulted in a mean secretory activity, defined as IPX, of -0.19 ± 0.02 that was statistically significant from nontreated control cells (one-tailed Student's *t*-test: $p \leq 0.001$). Cells treated with reconstituted freeze-dried MBC beverages showed a more positive IPX (-0.14 ± 0.04) than those from RC, although this result did not

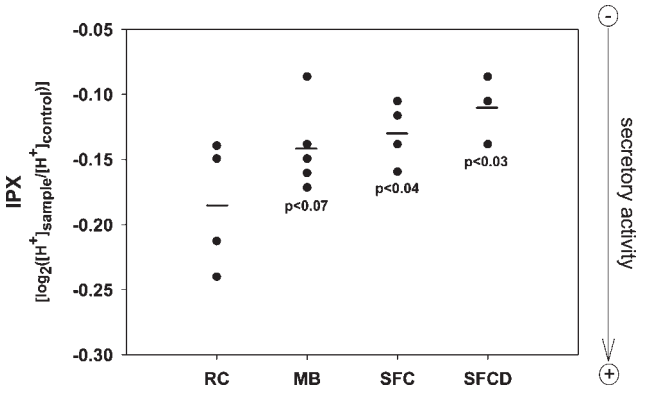

Figure 5. Intracellular proton index (IPX) of HGT-1 cells treated with reconstituted freeze-dried coffee beverages prepared from commercial coffee samples (regular coffee: RC, $n = 4$; mild bean coffee: MBC, $n = 5$; stomach-friendly coffee: SFC, $n = 5$; decaffeinated stomach-friendly coffee: SFCD, $n = 3$) in individual concentrations of 2.5 mg/mL for 10 min (single data points are displayed in the diagram, mean values are indicated by bars and were statistically evaluated by a one-tailed Student's *t*-test for MBC, SFC, or SFCD vs RC).

reach statistical significance (one-tailed Student's *t*-test: $p = 0.0744$). However, cells treated with reconstituted freeze-dried beverages prepared from SFC and SFCD did show significantly higher IPX values than those from RC (SFC: -0.13 ± 0.03; SFCD: -0.10 ± 0.02; one-tailed Student's *t*-test vs RC: $p \leq 0.05$), indicating higher intracellular proton concentrations and, thus, a lower proton secretory activity. These results demonstrate that technological processing applied to coffee beans in order to produce stomach friendly coffee or decaffeinated coffee actually does result in coffee beverages that show less proton secretory activities in human stomach cells. In other words, commercial extraction of raw coffee beans obviously removes compounds that promote the secretory activity. This result is in contradiction to a literature report of Fiebich et al. (*24*), who performed a patch-clamp technique in single stomach cells and did not find differences in the proton secretory activity among two RC and one steam-treated coffee samples. However, the small number of coffee samples studied by Fiebich et al. (*24*) might not have been sufficient to detect differences, which can also be seen from the results presented in this work, since some of the reconstituted freeze dried beverages prepared from SFC had comparable effects to those prepared from RC (**Figure 5**). Also, the most pronounced reduction in the proton secretory activity in the present study was demonstrated for SFCD.

Quantification of Candidate Stomach Irritating Components in Coffee and Correlation to the Proton Secretory Activity of Coffee Samples. In order to get first insights into the candidate molecules which might be responsible for the proton secretory activity of the coffee samples, coffee compounds putatively modulating the gastric acid and gastrin secretion (**Figure 1**), such as $^{\beta}N$-alkanoyl-5-hydroxytryptamides (C5HT, **1a**−**1f**), caffeine (**2**), *N*-methylpyridinium (**3**), chlorogenic acids (**4**), and some of their roasting products, e.g. caffeoyl quinides (**5**−**9**), pyrogallol (**10**), catechol (**11**), and 4-ethylcatechol (**12**), were quantitatively determined in brews obtained from customary coffee products (cf. **Tables 1** and **2**).

The total quantity of $^{\beta}N$-alkanoyl-5-hydroxytryptamides (C5HT), determined by stable isotope dilution analysis, was below < 0.5 mg/L and did not show substantial differences between regular coffee (RC), mild bean coffee (MBC), and stomach friendly coffee (SFC); for example, the means of the C5HT contents in the respective product category were rather close with 299.5 ± 134.1 μg/L (RC), 348.2 ± 95.2 μg/L (MBC), and

Table 2. Concentrations of Phenolic Coffee Constituents[a] in Brews Prepared from the Product Categories Regular Coffee (RC), Mild-Bean Coffee (MBC), Stomach-Friendly Coffee (SFC), or Stomach-Friendly Decaffeinated Coffee (SFCD)

		concentration (mg/L)								
sample		**4**	**5 + 6[b]**	**7**	**8**	**9**	**∑5−9**	**10**	**11**	**12**
RC	A	1433.3	80.9	14.8	4.3	53.7	153.7	3.49	4.54	0.91
	B	1318.1	63.1	18.1	5.1	36.2	122.5	5.12	8.66	1.15
	C	1228.6	88.3	18.7	4.9	57.3	169.2	4.04	7.25	1.08
	D	944.8	71.3	18.8	4.4	45.9	140.4	7.09	7.81	1.69
		1231.2 ± 208.5[a]					146.5 ± 19.8[a]	4.94 ± 1.59[a]	7.07 ± 1.78[a]	1.21 ± 0.34[a]
MBC	E	1118.7	79.2	17.2	5.3	51.2	152.9	5.46	7.47	1.52
	F	1254.4	69.9	15.7	3.9	53.2	142.7	3.72	5.68	0.94
	G	1260.9	79.9	19.1	4.8	50.4	154.2	4.58	7.16	1.38
	H	1113.6	83.1	17.3	4.4	56.6	161.4	5.90	7.08	1.52
	I	957.2	86.5	19.5	4.0	58.9	168.9	4.79	5.15	1.28
		1140.9 ± 124.8[a]					156.0 ± 9.8[a]	4.89 ± 0.84[a]	6.51 ± 1.03[a]	1.33 ± 0.24[a]
SFC	J	1445.9	86.5	19.5	4.0	58.9	168.9	3.70	4.54	1.14
	K	1197.3	85.2	19.5	4.5	57.8	167.0	4.80	5.19	1.32
	L	1252.1	67.0	16.4	3.7	43.2	130.3	5.42	6.43	1.58
	M	956.5	74.6	17.6	4.2	51.9	148.3	4.63	5.28	1.22
		1212.9 ± 201.5[a]					153.6 ± 18.1[a]	4.64 ± 0.71[a]	5.36 ± 0.79[a]	1.32 ± 0.19[a]
STCD	N	1298.6	70.5	19.0	4.6	50.7	144.8	3.98	5.11	1.13
	O	1187.6	70.8	17.8	4.7	50.1	143.4	4.82	5.10	1.44
	P	950.4	66.1	16.3	4.9	46.9	134.2	4.38	5.03	1.27
		1145.5 ± 177.9[a]					140.8 ± 5.8[a]	4.39 ± 0.42[a]	5.08 ± 0.04[b]	1.28 ± 0.15[a]

[a] Phenolic compounds are chlorogenic acids (**4**) given as the sum of 3-*O*-, 5-*O*-, and 4-*O*-caffeoyl quinic acids, caffeoyl quinides 5-*O*-caffeoyl-*muco*-γ-quinide (**5**), 3-*O*-caffeoyl-γ-quinide (**6**), 4-*O*-caffeoyl-*muco*-γ-quinide (**7**), 5-*O*-caffeoyl-*epi*-δ-quinide (**8**), and 4-*O*-caffeoyl-γ-quinide (**9**), as well as the hydroxybenzenes pyrogallol (**10**), catechol (**11**), and 4-ethylcatechol (**12**). Compounds **5** and **6** were quantified as the sum due to partial coelution. Different index letters in each row indicate significant differences between the respective product categories ($p < 0.05$).

336.2 ± 46.0 μg/L (SFC), respectively (**Table 1**). Also, the distribution of the individual C5HT derivatives was rather similar in the samples RC, MBC, and SFC, respectively, and $^{\beta}N$-arachinoyl-, $^{\beta}N$-behenoyl-, and $^{\beta}N$-lignoceroyl-5-hydroxytryptamide were found as the most abundant isomers, accounting for about 20−30%, 65%, and 10% of the C5HTs, respectively. On the basis of these data, neither steam treatment nor blending of "mild" beans, revealing low acidity of the final beverage, resulted in a significantly lower C5HT content in the resulting coffee brew.

In contrast, the decaffeination process had a significant impact (*t*-test, $p < 0.05$) on the C5HT content; for example, the brew prepared from steam treated-decaffeinated (SFCD) coffee (61.2−65.0 μg/L) showed about 5 times lower concentrations of C5HTs when compared to the regular product (299.5 ± 134.1 μg/L) (**Table 1**). Differing from the samples RC, MBC, and SFC, $^{\beta}N$-behenoyl- and lignoceroyl-5-hydroxytryptamide accounted for about 50 and 19% of the total C5HT content in the brew made from the SFCD sample, respectively, while $^{\beta}N$-arachinoyl-hydroxytryptamide remained unchanged (25−27%) when compared to the RC sample (**Table 1**).

Analysis of the alkaloid caffeine (**2**) also revealed no major differences between the beverages prepared from coffee of the category RC, MBC, and SFC, respectively; for example, caffeine contents ranged from 637.8 ± 51.6 (RC) to 688.1 ± 58.4 (MBC) to 735.7 ± 74.2 (SFC). As expected, the decaffeinated samples contained only small amounts of caffeine (SFCD 23.8 ± 9.9 mg/L; *t*-test $p < 0.001$) (**Table 1**).

The application of steam treatment did not lead to a coffee brew significantly depleted in *N*-methylpyridinium (NMP, **3**). Analysis of NMP by means of stable isotope dilution analysis revealed concentrations of 24.7 ± 9.4 mg/L in the brews prepared from RC samples and 24.7 ± 3.1 mg/L and 20.6 ± 2.1 mg/L in those from MBC and SFC samples. Interestingly, the decaffeinated stomach friendly coffee samples (SFCD) were analyzed for the lowest NMP concentrations of 15.5 ± 3.1 mg/L (**Table 1**).

Surprisingly, analysis of the chlorogenic acids (CQAs), given as the sum of the 3-*O*-, 5-*O*-, and 4-*O*-isomers, did not show remarkable differences among the coffee samples analyzed (**Table 2**). Independent of the product category group, concentrations between 1140 and 1230 mg/L were found, which is in contradiction to existing patent claims (*12*, *13*). The steam treatment used for the production of commercial coffee products obviously did not reduce the CQA content in coffee. In addition, quantitative analysis of caffeoyl quinides, formed upon thermal degradation of the chlorogenic acids during roasting, revealed only minor differences ($p > 0.05$) between the samples RC (146.5 ± 19.8 mg/L), MBC (156.0 ± 9.8 mg/L), SFC (153.6 ± 18.1 mg/L), and SFCD (140.8 ± 5.8 mg/L) (**Table 2**). Also, the ratio between the different isomers 5-*O*-caffeoyl-*muco*-γ-quinide (**5**), 3-*O*-caffeoyl-γ-quinide (**6**), 4-*O*-caffeoyl-*muco*-γ-quinide (**7**), 5-*O*-caffeoyl-*epi*-δ-quinide (**8**), and 4-*O*-caffeoyl-γ-quinide (**9**) was almost identical in all of the coffee brews investigated.

In addition, pyrogallol (**10**), catechol (**11**), and 4-ethylcatechol (**12**) originating from the thermal chlorogenic acid breakdown induced by coffee roasting (**14**) were determined by means of stable isotope dilution analysis (**Table 2**). Concentrations of pyrogallol (**10**) and 4-ethylcatechol (**12**) ranged from 3.49 to 7.09 mg/L and 0.91 to 1.69 mg/L. The beverages prepared from regular coffee, mild bean coffee, stomach friendly coffee, and steam

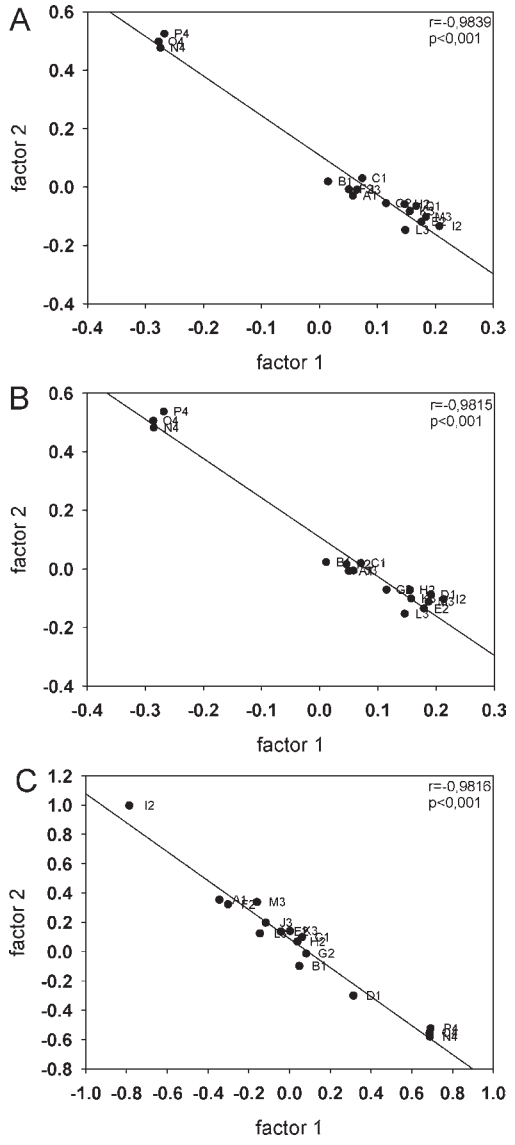

Figure 6. Principal component analysis (PCA). *Pearson* correlation coefficient and level of significance are displayed for each regression. (**A**) PCA including all quantified compounds for extraction of loading factors. (**B**) PCA including all quantified compounds for extraction of loading factors except $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides. (**C**) PCA including all quantified compounds for extraction of loading factors except caffeine.

treated-decaffeinated coffee showed comparable concentrations which did not differ significantly from each other; for example, 4.94 ± 1.59 mg/L (RC), 4.89 ± 0.84 mg/L (MBC), 4.64 ± 0.71 mg/L (SFC), and 4.39 ± 0.42 mg/L (SFCD) were found (**Table 2**). Interestingly, the concentration of catechol (**11**) was strongly dependent on the product category. For example, the highest concentrations of 7.07 ± 1.78 mg/L were found in regular coffees and mild bean coffees (6.51 ± 1.03 mg/L), followed by steam treated coffee (5.36 ± 0.79 mg/L) and SFCD-beverages containing the lowest amounts of 5.08 ± 0.04 mg/L (**Table 2**).

Taken together, the quantitative data indicate a significant impact of the processing technology applied to SFCD, resulting in the lowest concentrations of $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides, *N*-methylpyridinium, caffeine, and catechol.

Results from a principal component analysis (PCA) performed with the quantitative data from all of the commercial coffee samples tested revealed the strongest association for the decaffeinated coffee beverages (**Figure 6**A). Since it was of special interest whether $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides, *N*-methylpyridinium, caffeine, or catechol were mostly responsible for this result, PCA was performed with exclusion of either of these compounds. The first approach, excluding $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides, resulted in mostly similar factor loadings, such as those achieved when $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides were not excluded, indicating that it is very unlikely that $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides are the only components that are affected by the technological processes applied (**Figure 6**B). Similar results were obtained when catechol and *N*-methylpyridinium were excluded from PCA (data not shown). In contrast, when the PCA was performed without the quantitative data for caffeine, significantly different factor loadings, especially for coffee beverages prepared from decaffeinated coffee samples, were achieved (**Figure 6**C). However, despite exclusion of caffeine from PCA, an association of coffee beverages prepared from "decaffeinated coffee" samples, was still achieved. Therefore, we hypothesize that additional compounds besides caffeine, *N*-methylpyridinium, $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides, and catechol are substantially affected by processing technologies applied to produce "stomach friendly/decaffeinated coffee" (SFCD). This result is in agreement with the data obtained from cell culture experiments which showed a significantly lower impact of beverages prepared from SFC and SFCD samples on proton secretion in human stomach cells as compared to beverages prepared from regular (RC) and mild bean (MBC) coffee samples. SFCD samples differed from SFC samples in their contents of $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides, *N*-methylpyridinium, caffeine, and catechol, but not in their secretory activity on HGT-1 cells, indicating either effects by yet unknown components and/or synergistic effects thereof.

All together, the quantitative data collected revealed that steam treatment of coffee did not significantly affect the concentrations of any of the putative gastric acid secretion modifiers $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides (**1a**−**f**), caffeine (**2**), chlorogenic acids (**4**), caffeoyl quinides (**5**−**9**), pyrogallol (**10**), and 4-ethylcatechol (**12**) present in the SFC brew prepared.

Data on the intracellular proton index (IPX) clearly show that no single molecule out of the group of compounds, the contents of which were reduced by decaffeination and steam treatment, can be hypothesized to act as the key compound stimulating the proton secretion in human stomach cells. Next to the cell's treatment with SFCD, also that with SFC samples resulted in elevated IPX values compared to RC or MBC treated cells, but significantly lower contents of putatively stomach-irritating compounds compared to RC samples were only analyzed in reconstituted freeze-dried beverages prepared from SFCD. In consequence, either the synergistic effects of these compounds might affect the proton secretory activity, and/or yet unknown coffee ingredients need to function as potent inducers of acid secretion. Future studies will have to follow one or the other possibility in order to explain the acid secretory activity of coffee on a molecular level.

In conclusion, a novel approach to evaluate the gastric acid secretory activity of coffee beverages has been introduced and demonstrated to be suitable for the analysis of the proton secretory activity of coffee beverages. For the first time, quantitative data on the concentrations of $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides, the chemopreventive compound *N*-methylpyridinium, and the caffeoyl quinides 5-*O*-caffeoyl-*muco*-γ-quinide, 3-*O*-caffeoyl-γ-quinide, 4-*O*-caffeoyl-*muco*-γ-quinide, 5-*O*-caffeoyl-*epi*-δ-quinide, and 4-*O*-caffeoyl-γ-quinide in coffee beverages from customary coffee products claimed as stomach friendly are presented. The results indicate that, although the brews obtained from steam treated coffees show reduced proton secreting activity, the concentrations of the putatively irritating compounds

analyzed within this paper do not significantly differ from the respective concentrations in untreated, regular coffees. Brews prepared from solvent assisted decaffeinated and steam treated products significantly differed in the contents of the four compounds catechol, *N*-methylpyridinium, caffeine, and $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides, and they showed a significantly lower secretory activity compared to those of samples prepared from regular and mild bean coffees. However, this secretory activity was even reduced compared to those of only steam treated coffees, although the concentrations of those four compounds, catechol, *N*-methylpyridinium, caffeine, and $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides, were not different between steam treated and steam treated/decaffeinated coffees.

Correlation of the analytical and cell assay derived results challenges the coffee industries' hypothesis that chlorogenic acids and their breakdown products, catechol, 4-ethylcatechol, and pyrogallol, are the key players in stomach discomfort after ingestion of coffee brew. Further studies aimed at identification of compounds triggering anti- and prosecretory mechanisms of proton efflux in gastric cells as biomarkers of gastric acid secretion are currently under investigation.

ACKNOWLEDGMENT

This research project was supported by a grant from FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn, Germany), the AiF (Arbeitsgemeinschaft industrieller Forschung), and the German Ministry of Economics (Project No. 14042 N). The authors thank Dr. C. L. Laboisse (Inserm 94-04, Faculte de Medicine, Nantes) for kindly providing the HGT-1 cells, clone 6.

LITERATURE CITED

(1) Boekma, P. J.; Samsom, M.; van Berge Henegouwen, G. P.; Smout, A. J. Coffee and gastrointestinal function: facts and fiction. A review. *Scand. J. Gastroenterol. Suppl.* **1999**, *230*, 35–39.

(2) Cohen, S. Pathogenesis of coffee-induced gastrointestinal symptoms. *N. Engl. J. Med.* **1980**, 122–124.

(3) Nilsson, M.; Johnsen, R.; Ye, W.; Hveem, K.; Lagergren, J. Lifestyle related risk factors in the aetiology of gastro-oesophageal reflux. *Gut* **2004**, *53*, 1730–1735.

(4) Fodor, O.; Vestea, S.; Urcan, S.; Popescu, S.; Sulica, L.; Iencica, R.; Goia, A.; Ilea, V. Hydrochloric acid secretion capacity of the stomach as an inherited factor in the pathogenesis of ulcer. *Am. J. Dig. Dis.* **1968**, *13*, 260–265.

(5) Wendl, B.; Pfeiffer, A.; Pehl, C.; Schmidt, T.; Kaess, H. Effect of decaffeination of coffee or tea on gastro-oesophageal reflux. *Aliment. Pharmacol. Ther.* **1994**, *8*, 283–287.

(6) Pehl, C.; Pfeiffer, A.; Wendl, B.; Kaess, H. The effect of decaffeination of coffee on gastro-oesophageal reflux in patients with reflux disease. *Aliment. Pharmacol. Ther.* **1997**, *11*, 483–486.

(7) Buergin, E. Lowering the carboxylic acid-5-hydroxytryptamid content in unroasted coffee. *Ger. Off. Patent*, 1975, DE-74-2429233 19740618.

(8) Kurzhals, H.; Sylla, K. F. Eliminating irritative substances from coffee. Fr. Demande, 1978, FR 78-11492 19780419.

(9) Van der Steegen, G. Treatment of green coffee beans. *Ger. Off. Patent*, 1976, DE-6-76-2618545 19760428.

(10) Fehlau, R.; Netter, K. J. The influence of untreated and mild-treated coffee and carboxylic acid hydroxytryptamides on the stomach epithelium of the rat (in German). *Z. Gastroenterol.* **1990**, *28*, 234–238.

(11) Ehrlich, A.; Basse, H.; Henkel-Ernst, J.; Hey, B.; Menthe, J.; Lücker, P. W. Effect of differently processed coffee on the gastric activity difference and intragastric pH in healthy volunteers. *Methods Find. Exp. Clin. Pharmacol.* **1998**, *20*, 155–161.

(12) Lendrich, P.; Wemmering, E.; Lendrich, E. *Ger. Off. Patent*, 1933, DE1927L070519D 19271222.

(13) Darboven, A. Method for the quality improvement of raw coffee by treatment with steam and water (in German). *Europäisches Patentblatt*, 1997, 1997/05/95109295.6.

(14) Müller, C.; Lang, R.; Hofmann, T. Quantitative precursor studies on di- and trihydroxybenzene formation during coffee roasting. *J. Agric. Food Chem.* **2006**, *54*, 10086–10091.

(15) Farah, A.; De Paulis, T.; Trugo, L. C.; Martin, P. R. Effect of Roasting on the Formation of Chlorogenic Acid Lactones in Coffee. *J. Agric. Food Chem.* **2005**, *53*, 1505–1513.

(16) Farah, A.; Donangelo, C. M. Phenolic Compounds in Coffee. *Braz. J. Plant Physiol.* **2006**, *18* (1), 23–36.

(17) Frank, O.; Zehentbauer, G.; Hofmann, T. Bioresponse-guided decomposition of roast coffee beverage and identification of key bitter taste compounds. *Eur. Food Res. Technol.* **2006**, *222*, 492–508.

(18) Gaibel, J. P.; Wagner, C. A.; Caroppo, R.; Qureshi, I.; Gloechner, J.; Manuelidis, L.; Kirchhoff, P.; Radebold, K. The stomach divalent ion-sensing receptor SCAR is a modulator of gastric acid secretion. *J. Biol. Chem.* **2001**, *276*, 39549–52.

(19) Stadler, R. H.; Varga, N.; Hau, J.; Vera, F. A.; Welti, D. H. Alkylpyridiniums. 1. Formation in model systems via thermal degradation of trigonelline. *J. Agric. Food Chem.* **2002**, *50*, 1192–1199.

(20) Somoza, V.; Lindenmeier, M.; Wenzel, E.; Frank, O.; Erbersdobler, H. F.; Hofmann, T. Activity-guided identification of a chemopreventive compound in coffee beverage using in vitro and in vivo techniques. *J. Agric. Food Chem.* **2003**, *51*, 6861–6869.

(21) Rubach, M.; Lang, R.; Hofmann, T.; Somoza, V. Time-dependent component-specific regulation of gastric acid secretion-related proteins by roasted coffee constituents. *Ann. N. Y. Acad. Sci.* **2008**, *1126*, 310–314.

(22) Carmosino, M.; Procino, G.; Casavola, V.; Svelto, M.; Valenti, G. The cultured human gastric cells HGT-1 express the principal transporters involved in acid secretion. *Pflugers Arch.* **2000**, *440*, 871–880.

(23) Laboisse, C. L.; Augeron, C.; Couturier-Turpin, M. H.; Gespach, C.; Cheret, A. M.; Potet, F. Characterization of a newly established human gastric cancer cell line HGT-1 bearing histamine H2-receptors. *Cancer Res.* **1982**, *42*, 1541–1548.

(24) Fiebich, B. L.; Valente, P.; Ferrer-Montiel, A.; Candelario-Jalil, E.; Menthe, J.; Luecker, P. Effects of coffees before and after special treatment procedure on cell membrane activitys in stomach cells. *Methods Find. Exp. Clin. Pharmacol.* **2006**, *28*, 369–372.

(25) Wieder, E. D.; Hang, H.; Fox, M. H. Measurement of intracellular pH using flow cytometry with carboxy-SNARF-1. *Cytometry* **1993**, *14*, 916–921.

(26) Edwards, L. J.; Williams, D. A.; Gardner, D. K. Intracellular pH of the pre-implantation mouse embryo: effects of extracellular pH and weak acids. *Mol. Reprod. Dev.* **1998**, *50*, 434–442.

(27) Mitchell, S. L. Gastric secretion. *Curr. Opin. Gastroenterol.* **2004**, *20*, 519–525.

(28) Okabe, S.; Shimosako, K.; Amagase, K. Pharmacological regulation of gastric acid secretion in the apical membrane of parietal cells; a new target for antisecretory drugs. *J. Physiol. Pharmacol.* **2001**, *52*, 639–656.

(29) Robinson, M. Proton pump inhibitors: update on their role in acid-related gastrointestinal diseases. *Int. J. Clin. Pract.* **2005**, *59*, 709–715.

(30) Yao, X.; Forte, J. G. Cell biology of acid secretion by the parietal cell. *Annu. Rev. Physiol.* **2003**, *65*, 103–131.

(31) Lang, R.; Mueller, C.; Hofmann, T. Development of a stable isotope dilution analysis with liquid chromatography-tandem mass spectrometry detection for the quantitative analysis of di- and trihydroxybenzenes in foods and model systems. *J. Agric. Food Chem.* **2006**, *54*, 5755–5762.

(32) Stadler, R. H.; Varga, N.; Milo, C.; Schilter, B.; Vera, F. A.; Welti, D. H. Alkylpyridiniums. 2. Isolation and quantification in roasted and ground coffees. *J. Agric. Food Chem.* **2002**, *50*, 1200–1206.

(33) Lang, R.; Bardelmeier, I.; Weiss, C.; Somoza, V.; Hofmann, T. Quantitation of $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides in coffee by means of LC-MS/MS-SIDA and assessment of their gastric acid

secretion potential using the HGT-1 cell assay. *J. Agric. Food Chem.*, in press.
(34) Berglindh, T.; Obrink, K. J. A method for preparing isolated glands from the rabbit gastric mucosa. *Acta Physiol. Scand.* **1976**, *96*, 150–159.
(35) Durbin, B. P.; Hardin, J. S.; Hawkins, D. M.; Rocke, D. M. A variance-stabilizing transformation for gene-expression microarray data. *Bioinformatics* **2002**, *18*, S105–S110.
(36) Huang, S.; Yeo, A. A.; Gelbert, L.; Lin, X.; Nisenbaum, L.; Bemis, K. G. At what scale should microarray data be analyzed? *Am. J. Pharmacogenomics* **2004**, *4*, 129–139.

Received for review October 14, 2009. Revised manuscript received December 12, 2009. Accepted December 14, 2009.