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Alkylpyridiniums. 2. Isolation and Quantification in Roasted and Ground Coffees

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Recent model studies on trigonelline decomposition have identified nonvolatile alkylpyridiniums as major reaction products under certain physicochemical conditions. The quaternary base 1-methylpyridinium was isolated from roasted and ground coffee and purified by ion exchange and thin-layer chromatography. The compound was characterized by nuclear magnetic resonance spectroscopy (^{1}H, ^{13}C) and mass spectrometry techniques. A liquid chromatography−electrospray ionization tandem mass spectrometry method was developed to quantify the alkaloid in coffee by isotope dilution mass spectrometry. The formation of alkylpyridiniums is positively correlated to the roasting degree in *arabica* coffee, and highest levels of 1-methylpyridinium, reaching up to 0.25% on a per weight basis, were found in dark roasted coffee beans. Analyses of coffee extracts also showed the presence of dimethylpyridinium, at concentrations ranging from 5 to 25 mg/kg. This is the first report on the isolation and quantification of alkylpyridiniums in coffee. These compounds, described here in detail for the first time, may have an impact on the flavor/aroma profile of coffee directly (e.g., bitterness), or indirectly as precursors, and potentially open new avenues in the flavor/aroma modulation of coffee.

KEYWORDS: Roasted and ground coffee; trigonelline; 1-methylpyridinium; 1,4-dimethylpyridinium; dialkylpyridinium; liquid chromatography−mass spectrometry (LC-MS); nuclear magnetic resonance spectroscopy (NMR)

INTRODUCTION

Trigonelline is the second most abundant alkaloid in green coffee and reaches levels in *Coffea arabica* and *Coffea canephora* var. *robusta* from 7.9 to 10.6 g/kg and from 6.6 to 6.8 g/kg, respectively (*1*). *Coffea* species cannot be differentiated with certainty on the basis of trigonelline content alone, even though there is a tendency toward higher concentration levels of the quaternary base in *arabica* species.

A further salient point is that trigonelline, in contrast to caffeine, undergoes significant degradation during roasting to afford a number of volatile and nonvolatile products (*2*). Model system studies have shown that two major pathways of decomposition are evident: (1) decarboxylation to afford a 1-methylpyridinium intermediate with subsequent methyl rearrangement to yield volatile pyridine and alkylpyridines (*3*) and (2) demethylation to afford methyl and amine esters of nicotinic acid (*4*). Moreover, the chemical yields of the nonvolatile decomposition products are low, with nicotinic acid and its progenitor methyl nicotinoate, for example, accounting for only ∼7% (weight basis) of reacted trigonelline (*4*). Even though a plethora of nitrogenous volatile chemicals originating from trigonelline have been described (*2*), there is a lack of quantitative data. Furthermore, no detailed studies have been performed to date in coffee on the nonvolatile reaction products of trigonelline with the exception of nicotinic acid and its methyl and amine esters.

A recent model study conducted in our laboratory on the thermal decomposition products of trigonelline under conditions encountered during coffee roasting has identified 1-methylpyridinium cation as a major reaction product. A further observation was the simultaneous formation of dialkylpyridiniums, albeit at concentration levels ∼2 orders of magnitude lower than the monomethylated analogues (*3*).

This study addresses the validity of the model, namely, whether 1-methylpyridinium can be isolated and quantified in roasted and ground coffee, comparing also the kinetics of alkylpyridinium formation (*3*) and how it relates to the temperature of roasting of coffee.

MATERIALS AND METHODS

Materials and Reagents. Pyridine (anhydrous, 99.8%), 1,4-dimethylpyridinium iodide, and iodomethane-d_3 (99.5 at. % D) were from Aldrich (Buchs, Switzerland). Acetonitrile was from J. T. Baker (HPLC grade). Chelex 100 (Na^+ form, 50−100 mesh) was from Bio-Rad. TLC sheets (Polygrom SilG/UV, 20 × 20 cm), 0.20 mm, were from Macherey and Nagel (Düren, Germany). Solid phase extraction cartridges (CBA, 6 mL, 1 g, Mega Bond Elut) were obtained from Varian (Middelburg, The Netherlands). The HPLC column (GROMSIL 80 SCX, 5 μm, 50 × 2 mm i.d.) was purchased from GROM

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Table 1. 360.13 MHz ^{1}H NMR Data[a] in D_2O of Synthetic 1-Methylpyridinium Iodide, the Deuterium-Labeled Analogue, and 1-Methylpyridinium Isolated from Coffee

proton	synthetic iodide	synthetic 1-$^2H_3$ iodide	coffee isolate
N-CH_3	4.436 ppm, s, 3 H	4.412 ppm, sl. br., ≤ 0.005 H	4.406 ppm, s, 3 H
4-CH	8.570 ppm, "t", $J \sim 7.8$ Hz, 1 H	8.575 ppm, t t, $J \sim 7.9$, 1.3 Hz,[b] 1 H	8.542 ppm, "t", $J \sim 7.9$ Hz, 1 H
3,5-CH	8.090 ppm, "t" sl. br., $J_{av} \sim 6.9$ Hz, 2 H	8.094 ppm, "t" br., $J_{av} \geq 6.9$ Hz, 2 H	8.056 ppm, "t" sl. br., $J_{av} \sim 6.9$ Hz, 2 H
2,6-CH	8.827 ppm, d, $J \sim 5.8$ Hz, 2 H	8.829 ppm, "d d t (?)", $J \sim 6.8$, 1.3 Hz,[b] ~ 2 H	8.795 ppm, d, $J \sim 5.9$ Hz, 2 H

[a] Shift values are given in part per million from indirect TSP standard (see NMR Measurements), coupling constants J in hertz. The measurements were done without internal temperature regulation (probe temperature ~23 ± 0.5 °C). Multiplicity abbreviations for ^{1}H NMR: s = singlet, d = doublet, t = triplet, m = multiplet. Quotes indicate approximate description of the coupling multiplet, and sl. br. = slightly broadened signals. [b] Better definition of multiplet structure in the deuterated compound because of nonexisting coupling to the methyl group. Moderate Gaussian resolution enhancement was applied to determine the coupling constants of this compound.

Table 2. 90.56 MHz ^{13}C NMR Data[a] in D_2O of Synthetic 1-Methylpyridinium Iodide, the Deuterium-Labeled Analogue, and 1-Methylpyridinium Salt Isolated from Coffee

carbon	synthetic iodide	synthetic 1-$^2H_3$ iodide	coffee isolate
N-CH_3 or N-CD_3	51.15 ppm, q, br.	50.48 ppm, s, weak deuterium multiplet pattern, $^1J_{C-^2H} \sim 23.4$ Hz	50.97 ppm, q, br.
4-CH	148.19 ppm, d	148.22 ppm, d	148.13 ppm, d
3,5-CH	130.88 ppm, d	130.88 ppm, d	130.80 ppm, d
2,6-CH	147.96 ppm, d "1:1:1", $^1J_{C-N} \geq 8.3$ Hz	147.95 ppm, d br. "1:1:1", $^1J_{C-N} \geq 8.3$ Hz	147.89 ppm, d "1:1:1", $^1J_{C-N} \sim 8.6$ Hz

[a] Shift values are given in parts per million from indirect TSP standard (see NMR Measurements). The measurements were done without internal temperature regulation (probe temperature ≥ 24 °C, due to decoupler heating). Multiplicity abbreviations for ^{13}C NMR: s = quaternary carbon, d = CH, q = CH_3.

Analytik and HPLC GmbH, Herrenberg-Kayh, Germany. Non-isotope-labeled 1-methylpyridinium was synthesized and characterized as described (*3*). All other reagents and solvents were of analytical grade purity. D_2O and 3-trimethylsilyltetradeuteriopropionic acid sodium salt (TSP) were purchased from Dr. Glaser AG (Basel, Switzerland).

Coffee Samples. Coffee samples (100 g) (*arabica* from Columbia) were roasted using a benchtop Probat roaster at 180 °C. The roasting time was varied (5−8 min) to obtain different roasting degrees, characterized by the color test number (CTN) based on near-infrared (NIR) reflectance and conducted on a Colortest II instrument (Neuhaus Neotec, Germany). Values defining a range from low roast (CTN 120) to high roast (CTN 40) were obtained. The coffee samples were ground using a Ditting mill, setting 5.5.

Sample Preparation. Roasted and ground coffee (5.4 g) was suspended in a final volume of 100 mL of hot 10 mM HCl (>90 °C) and filtered over a gold filter (Bosch coffee machine). Typically, a 10 mL aliquot of the filtrate was diluted 10-fold with 10 mM HCl and fortified with 2 mL of 1-d_3-methylpyridinium iodide (0.2 mg/mL stock solution, 4 μg/mL in the final extract). A portion of the filtrate (1.5 mL) was centrifuged (Eppendorf) for 5 min at 10000 rpm. Then, 1 mL of coffee solution was loaded onto a CBA cartridge (1 g), preconditioned with methanol and water (each 5 mL). After penetration of the coffee solution, the cartridge was rinsed with water followed by methanol (each 5 mL). The analyte was eluted with 2 N formic acid in water (5 mL). This acid fraction was either injected directly (5 μL) or diluted 20-fold (v/v) with water prior to LC-MS analysis. Analytical samples with levels of alkylpyridiniums not within the dynamic range of the standard curve were diluted so as to achieve an acceptable analyte range.

Large-Scale Isolation of 1-Methylpyridinium from Roasted and Ground Coffee. Roasted coffee beans (*arabica*, CTN 70) were ground in a laboratory mill (Falling Number model 3303) to a particulate size of 0.5−1 mm. The fines (50 g) were suspended in 10 mM HCl (500 mL) and refluxed for 35 min. The slurry was left to cool (ice) and centrifuged at 2970*g* (Beckman Coulter Avanti J-25I, rotor JCA-10-500) at 5 °C for 15 min. The supernatant was carefully removed and filtered over GF/B glass fiber filters (Whatman) and the clear filtrate loaded onto a glass column (20 × 5.5 cm) filled with Chelex (100 g). The Chelex exchange resin in the column was prewashed with 2 L of H_2O. After preconditioning, the filtrate was slowly loaded onto the column and after penetration left standing for 20 min. The column was then washed with water (1500 mL) and methanol (300 mL), and the 1-methylpyridinium was eluted with 2 N formic acid in water (250 mL). The eluate was concentrated in vacuo at 45 °C to ~10 mL and passed over a Sep-Pak column (Carbograph, 1 g, Alltech). The filtrate was concentrated in vacuo at 45 °C to a small volume and the yellow "gummy" residue taken up in ethanol and acidified with a few drops of concentrated HCl. A precipitate formed upon cooling, which was separated by filtration (0.76 g) and did not contain visible amounts of 1-methylpyridinium as determined by TLC. The mother liquor was again concentrated and this procedure repeated twice to remove the nonsoluble material. Finally, the mother liquor was applied to TLC sheets, solvent ethyl acetate/methyl ethyl ketone/formic acid/water (5:3:2:1), and after chromatographic development 1-methylpyridinium was eluted with 20% formic acid in methanol. The eluate was concentrated in vacuo at 35 °C and dried by lyophilization, yielding ~12 mg of amorphous material. Thorough NMR (^{1}H, ^{13}C) and high-resolution mass spectrometry (HRMS) analyses were performed on this fraction. HRMS was performed on a Micromass QToF-2, which is a quadrupole mass spectrometer coupled to a high-resolution reflectron time-of-flight analyzer system, in the positive electrospray ionization mode (+ESI). Calibration was with PEG using ions *m*/*z* 89 and 133. Samples were dissolved in methanol/water (1:1, v/v) and infused at 10 μL/min. Calculated mass molecular ion = 94.0656; measured = 94.0655.

Synthesis of 1-(Methyl-d_3)pyridinium Iodide. Pyridine (0.5 g, 6.32 mmol) was added to ~0.5 mL of MeCN (dry, J. T. Baker). Iodomethane-d_3 (1 g, 6.9 mmol) was added dropwise. The mixture was kept for 30 min at room temperature (heat evolved) and heated for 15 min at 60 °C on a hot water bath. The oily yellow mixture was then cooled on ice, upon which immediate precipitation of the iodide salt occurred. The precipitate was brought to room temperature and washed with MeCN (room temperature), yielding 0.97 g (4.33 mmol, 68.5% yield based on pyridine) solid material (slight yellow color). The solid was then placed in a vacuum desiccator (P_2O_5): mp 116−117 °C [lit. 116−117 °C (*5*)]; high-resolution mass spectrometry, calcd mass molecular ion = 97.0845, measured = 97.0863. The ^{1}H and ^{13}C NMR data are shown in **Tables 1** and **2**.

Liquid Chromatography—Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS). A Micromass Quattro-LC (Micromass, Manchester, U.K.) quadrupole mass spectrometer was used in this study, equipped with a ZSpray electrospray ion source and coupled to a Waters 2690 Alliance separation module. HPLC separations were performed by ion exchange chromatography on a GromSiL 80 SCX column (5 μm, 50 × 2 mm i.d.), injecting 5 μL of sample. All runs were performed under isocratic conditions at a flow rate of 0.3 mL/min and using methanol/water 1:1 (v/v) containing a final concentration

of 50 mM ammonium acetate (pH not adjusted). The LC column was at room temperature. Typical retention times of 1-methylpyridinium and 1,4-dimethylpyridinium were 6.95 and 7.5 min, respectively.

MS conditions were as follows: instrument control and data processing were performed using MassLynx NT software (version 3.4). Operating parameters were as follows: positive ion mode, needle voltage typically set to 2.9 kV, cone voltage 32 V, and RF lens 0.15 V. The source block and desolvation temperatures were set at 140 and 380 °C, respectively. Nebulizer and desolvation gas flows were set to 97 and 704 L of N_2/h, respectively. The ion energy of the first and second quadrupole was 0.8 and 1.0 V, respectively. All data were acquired at a collision energy of 24 eV using argon as collision gas at a pressure of 0.25 Pa (1.9 mTorr). The dwell time was 0.2 s, the interchannel delay 0.03 s, and the mass span 0.1 D. Three single reaction monitoring (SRM) transitions were chosen for each compound as follows:

1-methylpyridinium, *m/z* 94.0 →
79.0*, 94.0 → 78.0, 94.0 → 67.0

1-d_3-methylpyridinium, *m/z* 97.0 →
79.0*, 97.0 → 78.0, 97.0 → 70.0

1,4-dimethylpyridinium, *m/z* 108 →
93.0*, 108 → 92.0, 108 → 65.0

For quantification purposes, transitions marked with an asterisk (*) were used.

NMR Measurements. The NMR measurements were conducted by dissolution of the compounds in 99.95% deuterated water (D_2O). No shift standard was added, and shift referencing was done by comparison with a 0.75% solution of TSP in D_2O measured under the same experimental conditions.

All NMR spectra were measured on a Bruker DPX-360 spectrometer equipped with a 5 mm quadrinuclear (QNP) probehead in a temperature-regulated laboratory at 23 ± 0.5 °C without further temperature stabilization. ^{1}H NMR spectra were acquired at 360.13 MHz, using a spectral width of 19.95 ppm, number of scans usually 64, 64K data points, an acquisition period of 4.56 s, and a relaxation delay of 10 s. The pulse duration was 8 μs, corresponding to ~67° pulse angle. ^{13}C NMR spectra were acquired at 90.56 MHz, using a level switched waltz16 proton decoupling pulse program with full decoupling during the 1.507 s acquisition period and 2 dB power level reduction during the 10 s relaxation delay. The spectral width was 240.04 ppm with 64K data points free induction decay (FID) size, and the pulse width 4 μs, also corresponding to ~67° pulse angle. Between 6664 (synthetic 1-$^2H_3$-iodide) and 20832 ^{13}C NMR transients (coffee isolate) were accumulated. Exponential line broadening of 0.05 Hz for proton spectra and 0.5 Hz for carbon spectra was applied before Fourier transformation.

Quantitation. Standards of 1-methylpyridinium iodide and 1,4-dimethylpyridinium iodide were prepared in Millipore-grade water from stock solutions stored at refrigerator temperature (0.2 and 0.02 mg/mL), and 1-methylpyridinium iodide was mixed with a fixed amount of the isotope-labeled internal standard (40 or 800 pg/μL, depending on level of fortification in the coffee extracts). For quantitation of the dialkylpyridiniums, an external calibration curve was established, and samples were not further diluted after the solid phase cleanup step. Calibration curves (five-point) were established in the concentration range from 0.2 to 2 ng/μL, and analytes not within the given range were diluted accordingly with water and re-injected. For 1-methylpyridinium, all data evaluation (using the MassLynx software) was normalized to the area response analyte (AN) to internal standard (IS), [AN_a/IS_a]. For all standard curves, $r^2 > 0.99$ was obtained, and the amount of the compounds in the samples was extrapolated from the respective linear regression equation. 1-Methylpyridinium and dimethylpyridinium concentrations are expressed as the free cations.

Statistical Evaluation. Data analyses of the mean values of 1-methylpyridinium in roasted and ground coffee were compared using one-way ANOVA followed by least significant difference (LSD).

RESULTS

Isolation of 1-Methylpyridinium from Roasted and Ground Coffee. Due to the cationic nature of the molecule, ion exchange chromatography was chosen as the method of extraction. Various ion exchange resins, for example, Dowex 50W-X8, were assessed, and Chelex 100 was found to be best with regard to recovery. Different volatile acids and combinations of acid strengths of the eluting solvent were tested, resulting in optimum conditions with 2 N formic acid, that is, recovery of >70%, determined by fortification of coffee with the deuterated cognate.

As judged by TLC analysis, further purification after the initial ion exchange step was necessary, and thus the fraction was concentrated and applied to silica gel sheets. Even though only relatively small amounts of material could be charged onto the sheets (0.25 mm thickness), these gave superior separation compared to the thicker TLC glass plates (0.5−1 mm). Elution of the quaternary amine, which was visualized under UV and by spraying the edges of the chromatogram with iodoplatinate reagent, was successful only with high acid strength in methanol (20% HCOOH, v/v), and initial attempts with HCl in methanol resulted in only partial elution of the target analyte. Although this procedure may potentially result in the coelution of plasticizers/binding agents originating from the TLC sheets, it provided a "cleaner" extract compared to fractions that were not subjected to this chromatographic step.

A number of attempts were made to directly crystallize 1-methylpyridinium as its chloride salt from the extracts, either directly after ion exchange or after the additional TLC step. In fact, the chloride salt could not be obtained in a solid form (also attempted with the standard compound), and the target compound was always present in the mother liquors, enabling removal of coextractives using HCl/ethanol. After multiple fractionations, the mother liquor (only one spot visible by TLC/UV) was analyzed by NMR for structural confirmation.

NMR Investigations. NMR analysis was analogous to the previous work on the 1-methylpyridinium synthesis and isolation from the pyrolysate mixture (*3*). The ^{1}H and ^{13}C NMR data of the 1-methylpyridinium salt isolated from coffee are shown in **Tables 1** and **2**, together with those of the deuterium-labeled cognate. The proton spectrum of the latter was found to be very similar to that of the synthetic iodide, except for the missing methyl proton signal, which proved a nearly quantitative deuteration (<0.5% proton equivalents left).

The coupling patterns of the deuterated compound were clearer than those of the undeuterated analogue, due to the lack of long-range couplings to the methyl group. The ^{13}C NMR data corroborated deuteration of the methyl group and were otherwise identical to that of the synthetic iodide. The methyl signal is expected as a seven-line multiplet, but only the five central lines could be clearly seen because of low signal/noise ratio. No signal of nondeuterated methyl groups was detected, whereas a small amount of dideuterated methyl cannot be excluded from the ^{13}C NMR spectrum, with an upper limit of ~0.5% given by the proton spectrum.

Quantification of 1-Methylpyridinium in *Arabica* Roasted and Ground Coffees of Various Degrees of Roasting. The isolation and characterization of chemical constituents in roasted coffee remain a challenge, mainly due to the natural product diversity and thus chemical complexity of coffee. LC-MS is a technique that in many cases does not require extensive cleanup of a food matrix, even when the analyst needs to approach trace levels (*6*). However, roasted coffee can be chemically defined as a very complex matrix, and thus trials with different

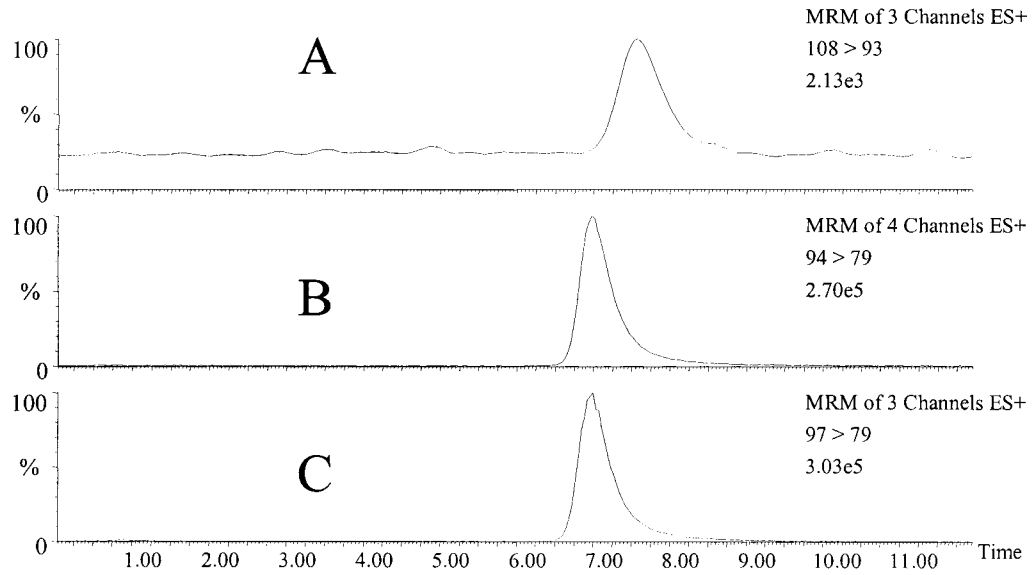


Figure 1. LC-ESI-MS/MS chromatogram of a roasted and ground coffee sample, CTN 105, showing the major mass transitions (SRM) for (A) dimethylpyridinium, (B) 1-methylpyridinium, and (C) 1-(methyl-d_3)pyridinium.

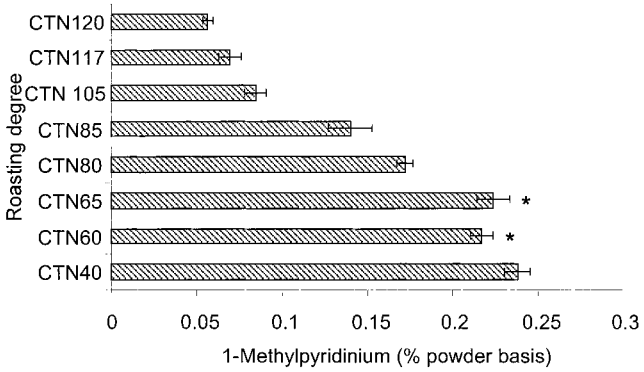


Figure 2. Quantitation of 1-methylpyridinium in coffees of various degrees of roast by isotope dilution LC-ESI-MS/MS. Values are averages of three independent determinations, each injected at least in duplicate. Asterisks indicate no statistically significant difference of the means at $\alpha = 0.01$.

commercially available ion exchange sorbents were conducted as a cleanup step prior to LC-MS.

Good recoveries of the analyte were achieved using a weak cation exchange resin (carboxypropyl). This solid phase allowed better recoveries of the analyte as compared to strong cation exchangers such as propylsulfonic acid, the latter requiring high acid strength for only partial desorption of 1-methylpyridinium from the resin. Best elution of the quaternary amine was achieved with 2 N HCOOH in water. Moreover, typical recoveries, determined from the area response of the labeled analogue in various coffee samples at a fortification level of 80 mg/kg, were 75.7 ± 5.7% ($n = 12$).

Optimization of LC-MS conditions is reported in the preceding study (*3*) and will thus not be elaborated further. All analyses were done using SRM and choosing three transitions for each compound for added analyte confidence. Typical SRM traces for 1-methylpyridinium, its stable isotope-labeled (methyl-d_3) analogue, and 1,4-dimethylpyridinium in a sample of a roasted and ground coffee are illustrated in **Figure 1**.

The analyses of a number of roasted and ground coffees of different roasting degrees were performed to determine a possible correlation of 1-methylpyridinium to the roasting degree. As shown in **Figure 2**, there exists a clear dependency of 1-methylpyridinium formation to temperature of roast, approaching up to 0.25% on a weight basis in the very dark roasted sample (CTN 40). Statistical treatment of the data confirms a good correlation of the roasting degree and 1-methylpyridinium levels, except between samples CTN 60 and CTN 65, which show no significant difference using 99% or 95% confidence levels. Good correlation of the variables is corroborated by the regression statistics, $r^2 = 0.957$.

Identification and Quantitation of Dialkylpyridinium in Roasted and Ground *Arabica* Coffees. The detection by LC-ESI-MS/MS of dialkylpyridinium in model systems (*3*) prompted the analysis of these compounds in coffee. Quantification is based on 1,4-dimethylpyridinium, and our preliminary investigations indicate that other alkyl substitution patterns leading to 1,2- and 1,3-dimethylpyridiniums may also be present; however, these could not be separated under the chromatographic conditions described here. As shown for 1-methylpyridinium, the dialkyl response shows significant correlation to the degree of roasting in the *arabica* coffees, determined in the same set of samples ($r^2 = 0.979$). As anticipated from the aforementioned data, a plot of the concentrations of the monoalkyl-/dialkylpyridinium reveals a good correlation (**Figure 3**), the latter reaching levels of up to 26 mg/kg in dark roasted coffee.

DISCUSSION

Very few studies exist to date on the natural occurrence of 1-methylpyridinium. The compound has been detected in various marine gastropods/bivalves, including crab, oysters, mussels, sea anemone (*7*), and possibly also squid (*8*). However, most of the data are only qualitative, and in some cases unequivocal identification, for example, by mass spectrometry, is lacking (*8*).

Pyridine/alkylpyridines are volatile constituents formed mainly in foods that are subjected to heat treatment and have been detected, for example, in heated corn oil (*9*), heated beef (*10*), roasted barley (*11*), and roasted coffee beans (*12*). A major contributor to the pyridine/alkylpyridine spectrum in roasted coffee is trigonelline, although it has been reported that proteins may also generate pyridine during pyrolytic degradation (*13*). However, no studies have been conducted on nonvolatile alkylpyridines, mainly because it is difficult to isolate and above all quantify azaheterocycles with a quaternary nitrogen.

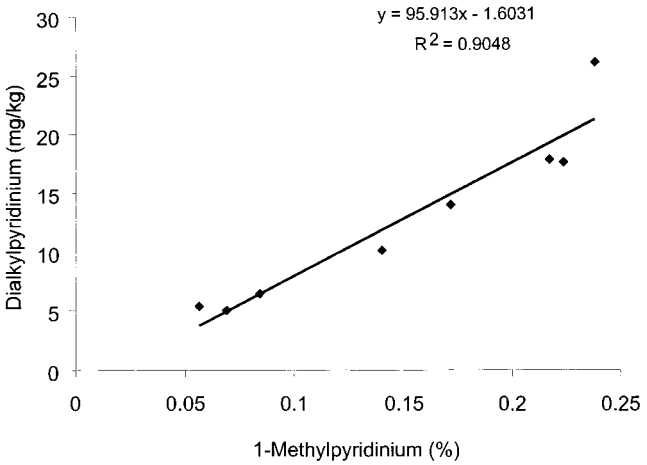


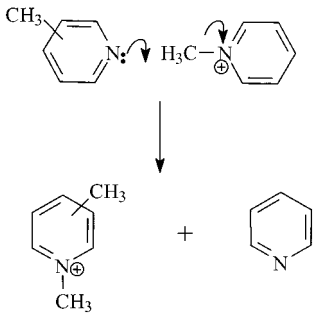
Figure 3. Correlation of 1-methylpyridinium/dialkylpyridinium, quantified as 1,4-dimethylpyridinium, in *arabica* roasted and ground coffees of various degrees of roast (CTN 40 → CTN 120). Plots are averages of duplicate determinations; quantitation of dialkylpyridinium is based on an external calibration curve constructed with 1,4-dimethylpyridinium iodide (data not corrected for recovery).

Our initial attempts to quantify alkylpyridiniums using HPLC with UV detection failed, even after a solid phase cleanup step, probably in part due to coffee coextractives that eluted with similar retention time under the given chromatographic conditions. Thus, chromatographic and MS conditions as described in a recent model system study were used for quantitation of the analytes in roasted coffee (*3*). This is the first report on the isolation, thorough chemical characterization, and quantification of 1-methylpyridinium in coffee. After discovery of this compound in model systems, we performed a literature review and found a very recent publication on high-resolution NMR investigations of reconstituted coffee in various solvents, which mentions the detection of 1-methylpyridinium in the whole coffee brew based on the characteristic chemical shifts in a ^{1}H spectrum (*14*). However, because this investigation was focused on NMR of the whole brew, no attempts were made to isolate or quantify the alkaloid in coffee.

The levels of 1-methylpyridinium in roasted and ground coffee are positively correlated to the degree of roasting, with a difference of close to 0.2% on a weight basis in light and dark roasted coffees. Even though we did not quantify trigonelline in the coffee samples, the ratio trigonelline/methylpyridinium could be determined and possibly used as an indicator of organic roasting loss, that is, an inverse relationship as described for the trigonelline:nicotinic acid ratio (*15*). As reported previously (*3*), dialkylpyridiniums have been identified as products of methyl rearrangement, involving methylpyridinium and nucleophiles such as alkylpyridine (**Scheme 1**). In fact, 1-methylpyridinium could potentially alkylate a number of other nucleophiles present in coffee, and investigations are now directed at important volatiles such as furfurylmercaptan, methylindoles, and alkylpyrazines. The latter may indeed lead to interesting nonvolatile pyrazinium cations (*16*), which may also exert an impact on the flavor/aroma profile of the brew.

An additional important role that methylpyridiniums may play is their direct contribution to coffee flavor. Caffeine accounts for only 10−30% of the total bitterness of roasted coffee brew (*17*, *18*), and the alkylpyridiniums (in particular the disubstituted pyridiniums) described in this paper may contribute to the bitterness of coffee. Recently, proline-based diketopiperazines were identified in roasted coffee and are suggested to play a role in the perceived bitterness of the brew, albeit without

Scheme 1. Proposed Reaction Pathway Leading to Dialkylpyridiniums in Coffee



quantitation of the compounds, which are probably present only in trace amounts (*19*).

Methylpyridiniums in roasted coffee must be associated with a negatively charged counterion, that is, either inorganic such as chloride or phosphate or even organic acids. Up to 38 different acids have been detected in roasted coffee, important organic ones, such as glycolic and lactic acid, being derived from carbohydrates (*20*). Yields of aliphatic acids such as formic, glycolic, and lactic increase with roasting but tend to become constant at higher roasting degree, probably due to volatility. A report in the literature indicates that *robusta* and *arabica* coffees show some differences in organic/inorganic acid yields during roasting (*21*). Phosphoric acid and quinic acid yields seem higher in *robusta*, the latter reflecting the chlorogenic acid content. Yields of other aliphatic acids (malic, formic, glycolic, and acetic) tended to be higher in *arabica*. It is possible that pyridiniums are associated with organic/inorganic acids and also with more complex polymers such as the melanoidins. Melanoidins are usually negatively charged at the pH of coffee, and model systems have shown that flocculation can be induced by reduction of repulsive electrostatic forces which can be brought about by inorganic cations (*22*). In this context, it may be important to investigate the impact of pyridinium cations on the behavior of melanoidins in model systems.

Further studies are also planned to determine the major structural isomer and ratio of the dialkylpyridiniums at different temperatures of roast and to investigate the potential formation of 3-ethyl- or 4-ethyl-1-methylpyridiniums in coffee. Such compounds could be of significance to the sensory attributes even if present only in low milligrams per kilogram levels, as their contribution to bitterness on a molar basis may be just as or more substantial than that of 1-methylpyridinium.

The present study reports the detection of substantial concentrations of alkylpyridiniums in roasted coffee. The major compound is 1-methylpyridinium, with levels from 0.06 to 0.25% on a weight basis in roasted and ground coffee. Taking into account the polarity of the molecule and therefore potentially the full extractability of 1-methylpyridinium into a coffee brew, then moderate coffee consumption (up to 5 cups/day) may lead to human exposures ranging from 30 to 125 mg/person/day of 1-methylpyridinium. The question of the health significance of such exposures is likely to be raised and has therefore to be addressed. With all of the available knowledge on coffee and health, both potential safety and beneficial aspects have to be evaluated.

Coffee is a complex mixture composed of >1000 different chemicals that occur at various levels in the product as consumed. However, the safety of such food products cannot be assessed on the basis of the toxicological information obtained with the individual constituents, some of which when

tested separately and at high concentrations may indeed have a toxicity in experimental systems. Safety judgments must rely on an evaluation of the mixture itself. Therefore, the safety of alkylpyridiniums present in coffee can be assessed indirectly using the data generated with coffee.

Coffee is a traditional food, with a long history of safe use in humans. In addition, coffee and coffee components have been the subject of many and extensive scientific investigations in both animal models and humans (*23*, *24*). Reviews of this information have indicated that evidence supporting a direct link between coffee intake and adverse health effects has been limited and inconsistent (*23*, *24*). On the contrary, human epidemiology strongly suggests the possibility for beneficial health effects such as a protection against colorectal cancer (*23*, *24*). Overall, the available scientific information supports the safety of moderate coffee consumption. This indirectly supports the safety of the alkylpyridiniums (including 1-methylpyridinium) as present in coffee at levels found in common brews. In addition, this raises the question regarding the possibility of beneficial health effects of alkylpyridiniums.

There are only very few data regarding the potential biological effects of alkylpyridiniums. 1-Methylpyridinium has been investigated in the context of the safety assessment of pyridine, a chemical widely employed in the pharmaceutical and chemical industries. In animal models and in humans, pyridine is biotransformed into various metabolites including 1-methylpyridinium (*25*). In an animal feeding study, 1-methylpyridinium was found to be less toxic than pyridine (*26*). However, other studies suggested that some of the pyridine effects, such as the induction of cytochrome P450 CYP1A1, could be attributed to *N*-methylpyridinium (*27*).

These results suggest that alkylpyridiniums, and in particular 1-methylpyridinium, may influence the expression and activity of xenobiotic metabolizing enzymes. Such a hypothesis is supported by data obtained with other structurally related pyridine derivatives such as picolines, which are known to increase the expression of various cytochromes P450 (*28*) and glutathione *S*-transferases (*29*). Coffee also shows similar effects (*30*). They are considered as potential mechanisms for its anticarcinogenic potentials (*24*, *30*). Because coffee is likely to be the major food source of 1-methylpyridinium, the role of this compound in the chemoprotective effects of coffee is under investigation in our laboratory.

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