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Alkylpyridiniums. 1. Formation in Model Systems via Thermal Degradation of Trigonelline

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Trigonelline is a well-known precursor of flavor/aroma compounds in coffee and undergoes significant degradation during roasting. This study investigates the major nonvolatile products that are procured after trigonelline has been subjected to mild pyrolysis conditions (220−250 °C) under atmospheric pressure. Various salt forms of trigonelline were also prepared and the thermally produced nonvolatiles analyzed by thin layer chromatography, liquid chromatography−electrospray ionization tandem mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance. Results revealed the decarboxylated derivative 1-methylpyridinium as a major product of certain salts, the formation of which is positively correlated to temperature from 220 to 245 °C. Moreover, trigonelline hydrochloride afforded far greater amounts of 1-methylpyridinium compared to the monohydrate over the temperature range studied. Investigations into other potential quaternary amine products of trigonelline also indicate nucleophilic substitution reactions that lead to dialkylpyridiniums, albeit at concentration levels ∼100-fold lower than those recorded for 1-methylpyridinium.

KEYWORDS: Trigonelline; nicotinic acid; 1-methylpyridinium; 1,4-dimethylpyridinium; alkylpyridinium; liquid chromatography−mass spectrometry (LC-MS); nuclear magnetic resonance spectroscopy (NMR); model system studies; pyrolysis

INTRODUCTION

The alkaloid trigonelline (1-methylnicotinic acid) (**Figure 1**) is fairly widely distributed in the plant kingdom, and its presence has been corroborated in a number of higher plant species such as the Lamiaceae and Asteraceae (*1*) and also in marine shellfish (*2*). Particularly high levels of trigonelline are found in green coffee beans, with typical levels reported for the various *Coffea* species and varieties ranging from 0.3 to 1.3% on a dry matter basis (*3*). The importance of trigonelline, as a precursor of flavor and aroma compounds as well as beneficial nutritional factors, is well documented in the literature (*4*, *5*).

The products of trigonelline resulting from thermal treatment were already addressed more than a half-century ago (*6*), which is not surprising given the abundance of the compound in green and roasted coffees. These initial studies demonstrated the formation of nicotinic acid when trigonelline is heated in a sealed tube. In fact, trigonelline decomposes via two major routes under typical temperatures as encountered during coffee roasting (220−250 °C), that is, (1) decarboxylation and methyl rearrangement to afford pyridines and (2) N-demethylation to give nicotinic acid (*6*).

The consumption of 3.5 standard cups of coffee per day contributes up to one-third of the minimum dietary requirement for an adult of nicotinic acid (*7*), a key product of trigonelline degradation and formed during roasting (*6*). Moreover, endogenous nicotinic acid in the green coffee bean is present at low concentrations (8−17 mg/kg) but is significantly augmented due to N-demethylation of the pyridine moiety of the progenitor, which is initiated at temperatures >180 °C (*5*).

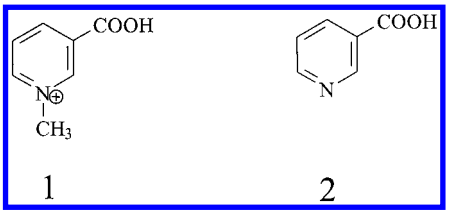

Figure 1. Chemical structures of trigonelline, **1**, and nicotinic acid, **2**.

There exist to date only very few mechanistic studies addressing the breakdown and conversion of trigonelline under mild pyrolysis conditions. The pathway of pyridine formation has been investigated in thermally treated trigonelline hydrate, a study that was based on deuterium exchange in alkylpyridines (*8*). Furthermore, in this model, nicotinic acid and pyridine were identified as the major reaction products, reaching 6.1 and 5.3% yields, respectively. An earlier study by Viani and Horman suggested the presence of relatively high amounts of methylnicotinoate, *N*-methylnicotinamide, and *N*,*N*-dimethylnicotinamide formed under their pyrolytic conditions (*4*), in contrast to only traces or low levels (<1%) of the same compounds measured in the more recent study (*8*). These discrepancies in levels of key reaction products may be due to the conditions of

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pyrolysis, for example, heating times, purity of the trigonelline salt, and quantification techniques used.

Despite these reports, there is clearly a lack of fundamental studies particularly with regard to the nonvolatile reaction products. This prompted us to characterize the major nonvolatile products of thermally treated trigonelline and to determine the impact of the nature of the trigonelline salt on the decomposition products formed as a function of both time and temperature. In the work presented here, liquid chromatography coupled to electrospray ionization tandem mass spectroscopy (LC-ESI-MS/MS) is mainly used to identify and quantify the major nonvolatile reaction products in a model system.

MATERIALS AND METHODS

Materials and Reagents. Trigonelline hydrochloride and nicotinic acid were purchased from Sigma (Buchs, Switzerland). Pyridine (anhydrous, 99.8%), 1,4-dimethylpridinium iodide, and iodomethane (99.5%) were from Aldrich (Buchs, Switzerland). Anion exchange resin AG 1-X8, 100−200 mesh, $^-$OH form, was from Bio-Rad, and Sil G/UV TLC plates from Macherey and Nagel. Silica gel plates $60F_{254}$ (1 mm) were from Merck. Iodoplatinate was purchased from Fluka. All other reagents and solvents were of analytical grade purity. D_2O and TSP (3-trimethylsilyltetradeuteriopropionic acid sodium salt) were purchased from Dr. Glaser AG, Basel, Switzerland.

Synthesis of 1-Methylpyridinium Iodide. Pyridine (2.5 g, 31.6 mmol) was added to ∼10 mL of MeCN (dry, J. T. Baker), and methyl iodide (5 g, 35.23 mmol) was added dropwise. The mixture was kept for 30 min at room temperature and heated for 10 min at 50 °C. The oily yellow mixture was then kept for 16 h at room temperature. Placement on ice resulted in immediate precipitation of the iodide salt. The precipitate was washed with MeCN (cold), dried in a desiccator under vacuum, and afforded 6.55 g (94% yield) of solid material (slight yellow color): mp 116−117 °C [lit. 116−117 °C (*9*)]; high-resolution mass spectrometry (HRMS): calcd mass molecular ion = 94.0656, measured mass molecular ion = 94.0656; ^{1}H NMR 4.436 ppm (s, 3 H, N−CH_3), 8.090 ppm ("t" sl. br., $J_{av} \sim 6.9$ Hz, 2 H, 3,5-H), 8.570 ppm ("t", $J \sim 7.8$ Hz, 1 H, 4-H), 8.827 ppm (d, $J \sim 5.8$ Hz, 2 H, 2,6-H); ^{13}C NMR 51.15 ppm (q, N−CH_3), 130.88 ppm (d, 3,5-CH), 147.96 ppm (d 1:1:1, 2,6-CH), 148.19 ppm (d, 4-CH).

Standard Pyrolysis Procedures. Typically, 50 mg of trigonelline, as either the hydrate, hydrochloride, or hydrogen sulfate salt (salts prepared by passage of trigonelline, 500 mg, through a column packed with 4 g of anion exchange resin, AG 1-X8), was heated on a heating module (Brouwer) in a tightly closed vacuum hydrolysis tube. After the predefined heating period, the tubes were allowed to reach room temperature and the residue was taken up in a small volume (2 × 2 mL) of 50% methanol/water (v/v). The tubes were sonicated to assist dissolution and the final volume made up to 5 mL with the same solvent. The samples were stored at −20 °C and diluted 500-fold with water prior to analysis by LC-ESI-MS/MS.

Large Scale Pyrolysis and Isolation of 1-Methylpyridinium Chloride. Trigonelline hydrochloride (500 mg, 3.64 mmol) was weighed in a Büchi round-bottom flask and 500 μL of water added at room temperature. The slurry was dried in a Büchi vacuum oven at 75 °C and then heated to 220 °C within ∼1.5 min. The compound was pyrolyzed at this temperature for a further 15 min. The residue was dissolved in 5 mL of water and applied to two C18 disposable cartridges (M&N, 1 g), preconditioned consecutively with 5 mL each of methanol, water, and 10 mM HCl. Each 2.5 mL of the reaction product was charged onto one column and eluted with 2 bed-volumes of water. The extract was concentrated in vacuo at 35 °C to remove excess solvent and lyophilized, affording in total 324 mg of water soluble material.

This fraction was then taken up in a small volume of methanol and acetonitrile added and left overnight at 5 °C. The crystals that formed were separated by filtration (identified as unreacted trigonelline by TLC), and the filtrate concentrated in vacuo at 40 °C. The residue was taken up in ethanol, acidified with concentrated HCl, and chromatographed by TLC on silica gel sheets in ethyl acetate/methyl ethyl ketone/formic acid/water (5:3:2:1). 1-Methylpyridinium ($R_f = 0.3$) was eluted with methanol containing 20% (v/v) formic acid; the eluate was concentrated in vacuo at 40 °C, and complete dryness was attained by subsequent lyophilization. This fraction (24 mg) was subjected to HRMS and NMR analysis: HRMS, calcd mass molecular ion = 94.0656, measured mass molecular ion = 94.0647; ^{1}H NMR 4.380 ppm (s, 3 H, N−CH_3), 8.031 ppm ("t" sl. br., $J_{av} \sim 6.9$ Hz, 2 H, 3,5-H), 8.516 ppm ("t", $J \sim 7.8$ Hz, 1 H, 4-H), 8.770 ppm (d, $J \sim 5.8$ Hz, 2 H, 2,6-H); ^{13}C NMR 50.94 ppm (q, broadened, N−CH_3), 130.77 ppm (d, 3,5-CH), 147.86 ppm (d 1:1:1, $^1J_{C-N} \sim 8.6$ Hz, 2,6-CH), 148.10 ppm (d, 4-CH).

High-Resolution Mass Spectrometry. High-resolution measurements were performed on a Finnigan MAT 8430 double-focusing mass spectrometer working in the electron ionization mode at 70 eV. The samples were directly introduced in the source heated at 180 °C. The resolution power was 5000, and perfluorokerosene was used as reference compound.

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 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 mmol/L ammonium acetate (pH ∼6.8, not adjusted). The column was at room temperature.

Instrument control and data processing were performed using MassLynx NT software, version 3.4 (Micromass). Operating parameters were as follows: positive ion mode, needle voltage typically set to 2.83 kV, cone voltage 32 V, and RF lens 0.15 V. The source block and desolvation temperatures were set at 140 and 400 °C, respectively. Nebulizer and desolvation gas flows were set to 90 and 710 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 Da. At least two single reaction monitoring (SRM) transitions were chosen for each compound as follows:

1-methylpyridinium, *m/z* 94 → 79*, 94 → 78, 94 → 67

1,4-dimethylpyridinium, *m/z* 108 → 93*, 108 → 92, 108 → 65

nicotinic acid, *m/z* 124 → 80, 124 → 78*

trigonelline, *m/z* 138 → 94, 138 → 92, 138 → 78*

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

NMR Measurements. The NMR samples were prepared by dissolution in 0.7 mL of 99.95% deuterated water (D_2O) and transferring the solution into a Wilmad 528-PP NMR tube with an outer diameter of 5 mm. No shift standard was added, and shift referencing was done by comparison with a 0.75% solution of 3-trimethylsilyltetradeuteriopropionic acid sodium salt (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 at ∼23 °C in a laboratory with regulated room temperature (±0.5 °C). ^{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, 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. Exponential line broadening of 0.05 Hz for proton spectra and 0.5 Hz for carbon spectra was applied before Fourier transformation. To confirm the assignments, a two-dimensional heteronuclear correlation experiment optimized for a 145 Hz one-bond proton−carbon coupling was done for one of the methylpyridinium salt samples, using a spectral width of 130.21 ppm for 8K data points

in the carbon dimension and 6.0 ppm for 256 data points in the proton dimension. An acquisition period of 0.347 s and a relaxation delay of 4.2 s were used. Sine square filtering was applied before two-dimensional Fourier transformation and the spectrum displayed and plotted in the magnitude mode. Quotes indicate approximate description of multiplets, sl. br. stands for slightly broadened, etc.

Quantitation. Standards were prepared in Millipore-grade water from a stock solution (0.2 mg/mL). External calibration curves (five-point) were established in the concentration range from 200 to 2000 pg/μL, and analytes not within the given range were adequately diluted with water and re-injected. For all standard curves, $r^2 > 0.99$ and the amount of the compounds in the samples was calculated from the respective linear regression equation.

RESULTS

Identification of 1-Methylpyridinium as a Major Product of Trigonelline Pyrolysis. Earlier reports on the thermal degradation of trigonelline have revealed nicotinic acid and nicotinamide, as well as their *O*- and *N*-methyl analogues, as reaction products (*4*, *8*). Thus, a first step in this study was to identify the major nonvolatile products formed during pyrolysis of trigonelline hydrochloride at temperatures ranging from 220 to 250 °C. The crude pyrolysates in methanol were subjected to TLC on silica gel sheets in a number of different solvent systems, spraying the sheets with iodoplatinate reagent. A prominent compound that did not correspond in its R_f value to the commonly known trigonelline breakdown/conversion products was detected in all pyrolysates, at various relative amounts over the whole temperature range. This compound reacted as an intense dark blue spot and migrated in a relatively acidic solvent (ethyl acetate/methyl ethyl ketone/formic acid/water, 5:3:2:1), with an R_f at ~0.3.

Compounds with a quaternary amine function show intense coloration with iodoplatinate reagent, indicating that this major reaction product has retained the *N*-methyl group. Large-scale pyrolysis was conducted with more starting material (0.5 g of trigonelline) and the extract purified by TLC in the same solvent system as described above, eluting the compound with 20% formic acid in methanol. Preliminary mass spectral analysis by direct infusion ESI-MS/MS in the positive mode suggested a 1-methylpyridinium salt, with a peak at *m/z* 94 [M^+], corresponding to the molecular formula C_6H_8N. This approach also enabled the detection of additional azaheterocycles under certain reaction conditions, identified as dimethylpyridinium salts, substituted with a methyl group at either the 2-, 3-, or 4-position of the pyridinium moiety. However, due to the paucity of material and relatively low concentrations of the dialkylpyridiniums, no attempts were made to isolate and further identify the compound(s) by NMR techniques.

Thorough NMR analysis of the pyrolysis product was done in D_2O. The ^1H and ^{13}C data of synthetic 1-methylpyridinium iodide were compared with methylpyridinium isolated from the pyrolysate. Both compounds show the classical AA′BB′C spin system of a pyridine, where the AA′ signals are slightly broadened due to the neighboring quadrupolar ^{14}N atom, and an *N*-methyl signal, which is also slightly broadened. The shifts of the signals are very similar for the synthetic and the pyrolysis products, the only significant difference observed being the much broader water signal (HDO, ~4.81 ppm) in the methylpyridinium isolated from the pyrolysate, probably due to a different pH of the latter.

Analogous findings are also reflected in the carbon-13 spectra. Here, the signals of the proton-decoupled 2,6-CH carbons showed a resolved triplet structure due to the one-bond coupling with the quadrupolar ^{14}N nucleus. The intensity ratio of the triplet was ca. 1:1.9:1. This does not represent a "classical" 1:2:1 triplet, as would be caused by coupling with two equivalent protons, but it is in fact a quadrupolar 1:1:1 pattern ($^1J_{C-N} \geq 8.3$ Hz), where the outer lines contribute to the intensity of the center line because of the significant line width. This pseudo-triplet structure was also present on the signals of the methyl but was not resolved, because the coupling constant is smaller. The ^1H NMR, ^{13}C NMR, and HRMS data thus confirm 1-methylpyridinium ion as a major product of trigonelline breakdown in this model system.

Quantification of the Major Nonvolatile Transformation Products of Trigonelline Hydrochloride. *LC-ESI-MS/MS.* A major challenge at the onset of this study was to achieve a separation of the starting material and the major pyrolysis products, particularly 1-methylpyridinium ion, in one single analytical run. Thus, different LC stationary phases/columns were assessed, ranging from C18 to various cation exchange resins. Unfortunately, the quaternary base could not be retained on the C18 columns tested but showed retention on a strong cation exchange column, under isocratic conditions with 50 mM ammonium acetate (pH not adjusted) in methanol (50%, v/v). Our first approach was to use conventional LC coupled to UV detection, but this revealed impurities in most fractions at the retention time of 1-methylpyridinium. Therefore, to achieve quantification of the alkylpyridiniums and nicotinic acid in a single run, separation/detection was done by LC-ESI-MS/MS.

Under positive ESI conditions, 1-methylpyridinium and 1,4-dimethylpyridinium show abundant M^+ ions without fragmentation. Because *N*-alkylpyridiniums are already charged in solution, they are amenable to ionization directly from the liquid phase. The daughter ion spectra obtained upon dissociation of the M^+ ion of 1-methylpyridinium and 1,4-dimethylpyridinium are portrayed in parts A and B of **Figure 2**, respectively. Details of fragmentation for the compounds studied here have been reported previously (*10*), and only salient features for the alkylpyridiniums will be mentioned. The main fragmentation in positive ESI-MS of 1-methylpyridinium is the loss of the methyl group to afford *m/z* 79 $[C_5H_5N]^{\bullet+}$ and *m/z* 78 $[C_5H_4N]^+$. Ring opening of the pyridinium moiety with retention of the *N*-methyl group affords the fragment *m/z* 67 $[C_5H_7]^+$. A characteristic transition for the dialkylpyridinium is the $[M - 1]^+$ ion at *m/z* 107. The fragmentation pattern also shows $[C_6H_6N]^+$ at *m/z* 92 (loss of a methyl group) and loss of HCN to afford *m/z* 65 $[C_5H_5]^+$.

In this study, ESI-MS/MS was the method of choice to quantify the aforementioned compounds, with emphasis on chromatographic separation of the quaternary amine base 1-methylpyridinium salt. Potential ion suppression by coelution of matrix constituents was largely avoided due to the strong dilution of the fractions, and thus quantification was done by establishing external calibration curves. All analyses were done using SRM and choosing three transitions for each compound of interest for certainty of the analyte (i.e., responses in all three transitions and ion ratios comparable to the standards). Typical SRM traces detecting the aforementioned two nonvolatile products nicotinic acid and 1-methylpyridinium of pyrolyzed trigonelline (240 °C) are illustrated in **Figure 3**.

Impact of Temperature. A series of experiments were designed to follow the formation of 1-methylpyridinium and nicotinic acid (a major N-demethylated product) at different temperatures and over time (**Figure 4**).

Three independent determinations were conducted with trigonelline hydrochloride as the starting material, which was heated under atmospheric conditions in tightly closed hydrolysis

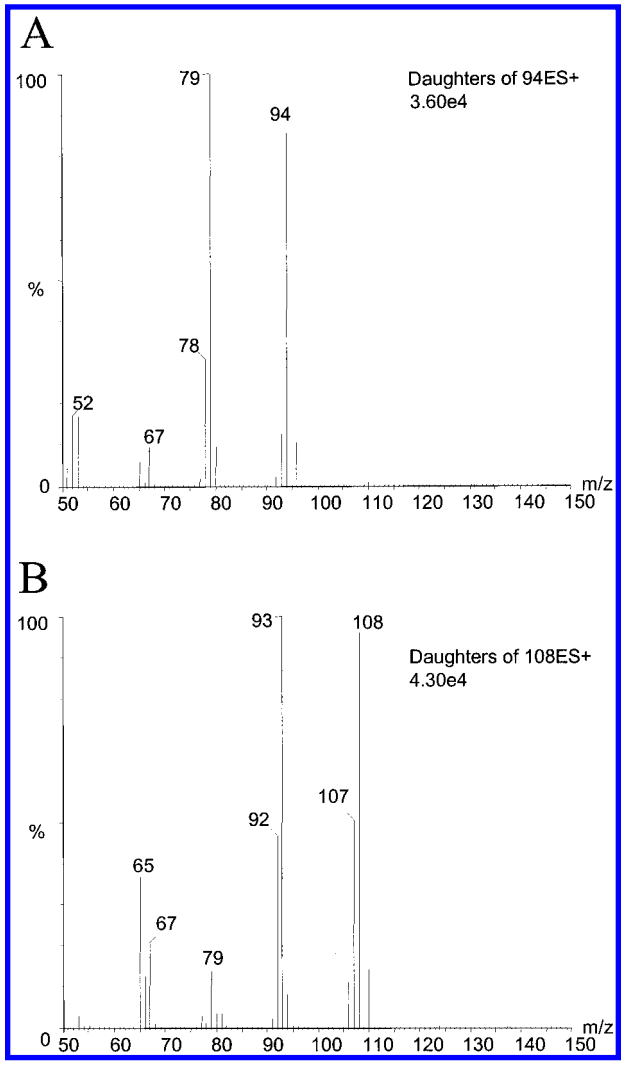


Figure 2. ESI-MS/MS daughter ion spectra of (A) 1-methylpyridinium cation and (B) 1,4-dimethylpyridinium cation.

tubes over the temperature range of 220−250 °C, in 5 °C increments, with a heating period of 15 min. After thermal treatment, the residue was dissolved in 50% methanol/water (v/v) and a strongly diluted aliquot analyzed by LC-ESI-MS using the conditions described. The amount of 1-methylpyridinium formed during thermal treatment of trigonelline is strictly dependent on temperature, increasing from 220 to 245 °C and then decreasing suddenly at 250 °C (**Figure 4**).

Interestingly, the maximum amount of the decarboxylated product was always measured at 245 °C (five independent experiments conducted over the same temperature range), with excellent correlations between independent experiments ($r^2 > 0.99$). An individual plot of temperature versus natural log amount of 1-methylpyridium formed shows a correlation of $r^2 = 0.975$ up to 245 °C. However, the rate equation(s) leading to 1-methylpyridinium may be rather complex, and kinetics would have to take into account methyl rearrangements and radical-based mechanisms (*4*). In this context, methyl groups attached to quaternary nitrogens are potential alkylating agents, and thus we included in our analyses the determination of 1,4-dimethylpyridinium, which could be procured by nucleophilic displacement (*11*). Notably, the transitions detected could originate from a number of methyl substitution patterns, that is, 1,2-, 1,3-, and/or 1,4-dimethylpyridinium. Under the LC conditions employed, no separation of the aforementioned structural isomers was possible. All three compounds, synthesized either in our laboratory or obtained commercially, showed identical daughter ion spectra (data not shown).

Dimethylpyridinium could be detected in most of the samples based on LC-ESI-MS/MS (using three SRM transitions, *m*/*z* 108 → 93, *m*/*z* 108 → 92, and *m*/*z* 108 → 65, and comparable ion ratios) and eluted from the LC column ∼0.5 min later than 1-methylpyridinium. Even though the yield of the dialkyls in the pyrolysis experiments was in all cases significantly lower than the monoalkyl derivative (by a factor of ∼100), it nevertheless was detected at various levels in many of the samples and showed a positive correlation to the amount of 1-methylpyridinium formed in this model system (**Figure 5**).

A route relatively well described in reports on the fate of trigonelline during pyrolysis or during the roasting of coffee is N-demethylation of the progenitor to afford nicotinic acid, which is positively correlated to the degree of roasting and does not decompose per se at elevated temperatures (*5*, *6*, *12*). Using

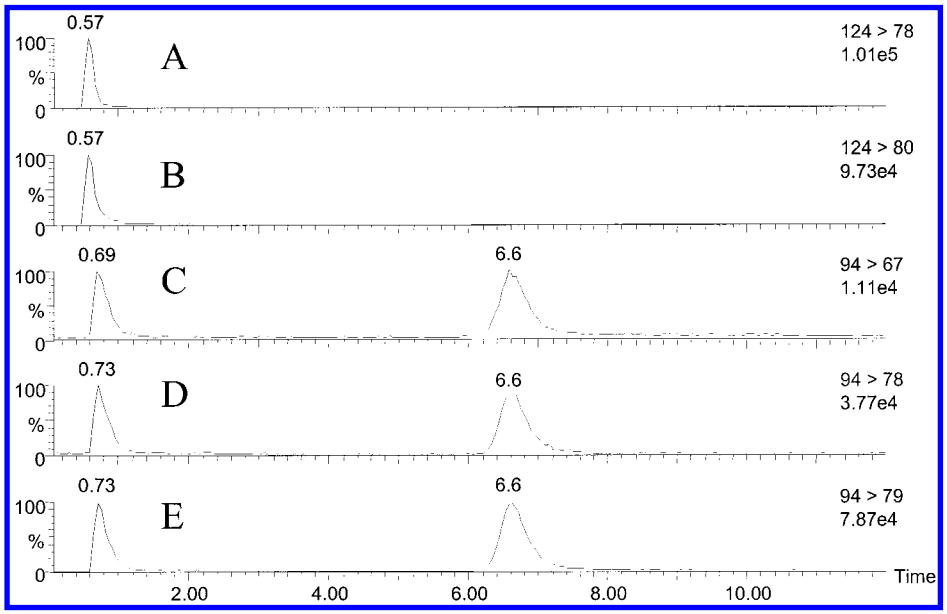


Figure 3. LC-ESI-MS/MS chromatogram of pyrolyzed trigonelline hydrogen chloride, using transitions (SRM) for nicotinic acid (A, B, $t_r = 0.6$ min.) and 1-methylpyridinium (C–E, $t_r = 6.6$ min) as described in the text.

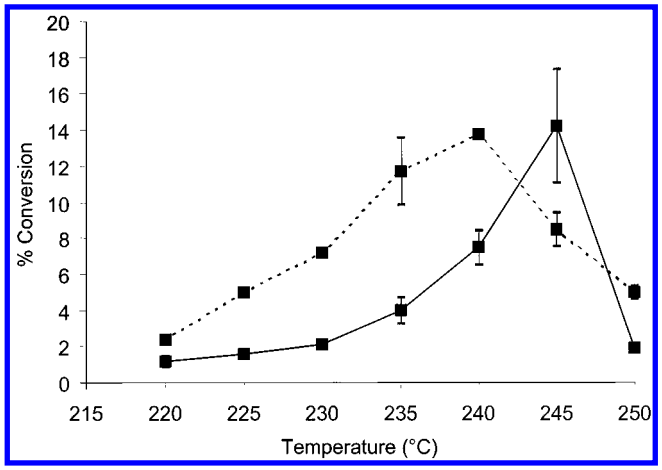


Figure 4. Formation of nicotinic acid (dashed line) and 1-methylpyridinium (solid line) from trigonelline hydrogen chloride as a function of temperature, expressed as percent conversion on a molar basis. Pyrolysis time = 15 min. Entries are averages of three independent determinations, depicting ± SD.

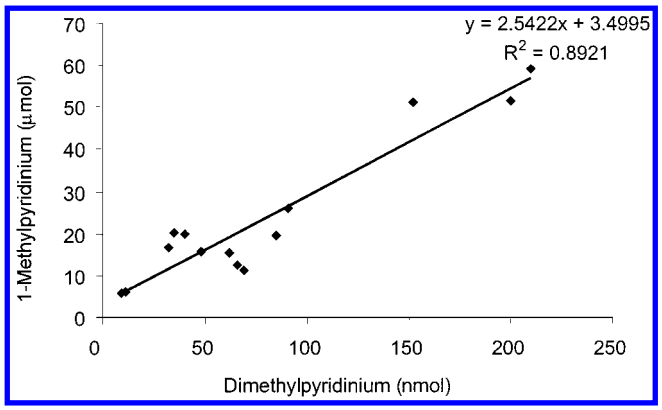


Figure 5. Plot of the ratio of the amounts of the quaternary amine reaction products 1-methylpyridinium/dimethylpyridinium, obtained after pyrolysis of trigonelline hydrochloride at 240 °C over various time periods ranging from 5 to 45 min.

the SRM trace *m*/*z* 124 → 78, nicotinic acid could be quantified in all experiments even though it showed no retention on the SCX column under the conditions used (t_r = ~0.6 min). Three independent experiments with trigonelline hydrochloride showed a maximum conversion to nicotinic acid at 235−240 °C, with a prominent decrease above this temperature (**Figure 4**). This indicates that N-demethylation is favored at slightly lower temperatures versus decarboxylation, probably due to the quaternary nitrogen that favors methyl transfer reactions, to yield methyl nicotinoate, for example. Furthermore, the decomposition of trigonelline over the temperature range 220−240 °C showed only a relatively slight decline (from 46.6 to 37.5% of trigonelline remaining, expressed on a molar basis) in the temperature range 220−240 °C, with prominent degradation of the starting material measurable at temperatures >240 °C (5−7% of starting material remaining at 245 °C), which is in accord with data published in the literature (*12*).

Formation of Major Reaction Products over Time. In all of the experiments described above, a fixed time point, that is, 15 min, was chosen over a variable temperature range. To determine the formation of the key reaction products as a function of time, trigonelline hydrochloride was heated at 230, 240, and 250 °C at six different time points, that is, 5, 10, 20, 30, 45, and 60 min (all data are averages of three independent determinations). Moreover, 41 and 38% of the starting material could be recovered after 1 h of thermolysis at 220 and 230 °C, respectively, demonstrating that trigonelline is relatively stable over an extended time. Trigonelline decomposition was accelerated at relatively higher temperatures (240 °C), with 94% of the starting material decomposed after 45 min. Thus, trigonelline breakdown is markedly increased at temperatures of ≥240 °C, as already observed in the experiments over different temperature ranges.

Table 1. Formation of 1-Methylpyridinium from Trigonelline Hydrochloride Heated at 220, 230, and 240 °C as a Function of Time, Determined by LC-ESI-MS/MS[a] and Expressed as Percent Conversion on a Molar Basis[b,c]

pyrolysis time (min)	220 °C	230 °C	240 °C
5	0.64 ± 0.1	1.14 ± 0.14	2.53 ± 0.8
10	1.33 ± 0.05	1.77 ± 0.16	6.63 ± 0.67
20	1.63 ± 0.27	3.08 ± 0.53	18.79 ± 1.55
30	1.77 ± 0.28	3.18 ± 0.13	4.56 ± 0.74
45	1.29 ± 0.28	2.18 ± 0.06	7.12 ± 1.8
60	1.08 ± 0.21	1.77 ± 0.61	9.15 ± 1.79

[a] Transition *m*/*z* 94 → 79. [b] Based on the assumption that upon 100% conversion, 0.287 mmol of trigonelline may afford 0.287 mmol of 1-methylpyridinium (determined as the free cation). [c] Entries are averages ± SD of *n* = 3 independent determinations.

Table 2. Formation of Nicotinic Acid from Trigonelline Hydrochloride Heated at 220, 230, and 240 °C as a Function of Time, Determined by LC-ESI-MS/MS[a] and Expressed as Percent Conversion on a Molar Basis[b,c]

pyrolysis time (min)	220 °C	230 °C	240 °C
5	0.35 ± 0.13	1.47 ± 0.27	5.26 ± 0.72
10	1.56 ± 0.27	5.1 ± 0.96	14.1 ± 0.31
20	4.34 ± 0.77	12.75 ± 0.83	16.85 ± 2.74
30	6.86 ± 0.73	14.26 ± 1.0	11.36 ± 0.46
45	9.3 ± 1.16	15.27 ± 0.35	10.0 ± 0.88
60	10.85 ± 0.73	17.08 ± 0.83	6.7 ± 1.46

[a] Taking transition *m*/*z* 124 → 78. [b] Based on the assumption that upon 100% conversion, 0.287 mmol of trigonelline may afford 0.287 mmol of nicotinic acid. [c] Entries are averages ± SD of *n* = 3 independent determinations.

The generation of 1-methylpyridinium and nicotinic acid under conditions as described above over time and temperature is shown in **Tables 1** and **2**, respectively. The formation of the quaternary base reaches a maximum after 20 min over all three temperature ranges, positively correlated to the temperature of pyrolysis as illustrated in the temperature−time-dependent measurements. At the highest temperature in the series (240 °C), 1-methylpyridinium reached 18% conversion in an experiment with three independent determinations, showing a steep incline up to 20 min (**Table 1**). This is also reflected by the data of **Figure 4**, which shows a maximum decarboxylation at 245 °C to yield close to 15% 1-methylpyridinium on a molar basis (note, in this case the heating time was 15 min). Prolonged heating at 240 °C (>30 min) resulted in a significant decrease in 1-methylpyridinium levels, probably due to further decomposition and/or interaction with other thermolysis products. In contrast to the temperature−time pattern observed for 1-methylpyridinium, nicotinic acid formation follows a clear positive correlation at 220 and 230 °C over the measured time period of 1 h (**Table 2**).

Reaction rate plots [ln $C_{100}/(C_{100} - C_t)$ versus time, where C_{100} = moles of nicotinic acid at 100% conversion, and C_t represents the amount formed at time *t*] were calculated over

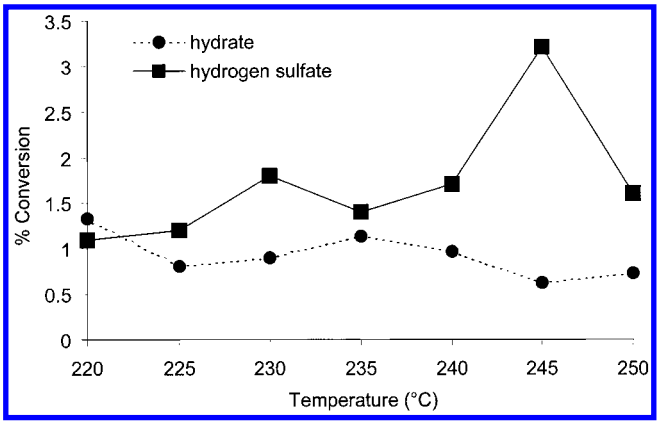


Figure 6. Yield of 1-methylpyridinium from trigonelline salts (hydrate, hydrogen sulfate) as a function of temperature, expressed as percent conversion on a molar basis. Pyrolysis time = 15 min. All entries are averages of duplicate determinations.

the first 20 min and showed good correlation ($r^2 = 0.9997$ and 0.9988 at 230 and 220 °C, respectively). The rate $\Delta C/\Delta t$ determined from the slope was ≈3-fold higher at 230 °C compared to that at 220 °C. The kinetics of nicotinic acid formation do not, however, continue to show linearity after 20 min with a significant decrease particularly at 240 °C, indicating that at this temperature other reaction routes may be preferred over simple demethylation of trigonelline or its *O*-methyl ester methyl nicotinoate.

Impact of the Trigonelline Salt on the Reaction Products Formed during Thermal Treatment of Trigonelline. Trigonelline hydrochloride showed a very distinct pattern of degradation over the temperature range studied, and the question addressed in this section is the potential impact of various salts on the degradation pathway of this compound. To our knowledge, none of the published studies on thermal degradation products of trigonelline take into account the possible ionic interaction with salts and how these could affect the product profile (*4*, *8*).

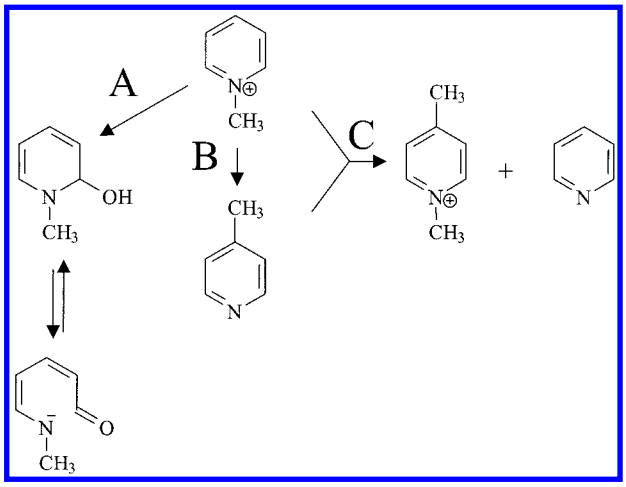
Commercially available trigonelline hydrochloride was converted to the hydrate by passage over an anion exchange column. Trigonelline hydrate was then subjected to thermal treatment over the same temperature range as described for the hydrochloride, and the reaction products 1-methylpyridinium and nicotinic acid were quantified by LC-ESI-MS.

Trigonelline hydrate revealed far less stability over the temperature range studied than the corresponding hydrochloride, with only ~3% of the starting material after a 15 min heating period at 220 °C. In addition, there was no further loss of trigonelline hydrate at relatively higher temperatures of 250 °C, which suggests that most of the hydrate was degraded already at relatively low temperatures. Advanced degradation/polymerization was reflected by a tarry black residue with a "sticky" consistency that was difficult to dissolve in organic solvents even after 30 min of sonification.

Salient differences were observed upon comparison of the yields of 1-methylpyridinium (**Figure 6**). Exchange of hydrogen chloride with hydrogen sulfate by passage of trigonelline through an anion exchange column shows accelerated decomposition to form 1-methylpyridinium at temperatures >225 °C. Also characteristic in this case is a clear increase of 1-methylpyridinium in the range 240−250 °C, analogous to the tendency observed for trigonelline hydrogen chloride (**Figure 4**), albeit with lower yield of the quaternary base.

The nature of the salt also has a pronounced impact on the formation of nicotinic acid. We observed that the conversion

Scheme 1. Proposed Reaction Pathways Leading to the Formation of Alkylpyridiniums



rates to nicotinic acid of the hydrate and sulfate salts are comparatively decreased, versus the hydrochloride (see **Figure 4**), over the same temperature range, yielding on a molar basis approximately 1% and 3.5% nicotinic acid at 235 and 245 °C, respectively.

DISCUSSION

This is the first report on the identification and quantification of the quaternary base 1-methylpyridinium as a major reaction product of trigonelline subjected to pyrolytic conditions. This quaternary base is formed by decarboxylation and, as demonstrated in this study, represents an important stable product/intermediate in the thermal reaction pathway(s) of trigonelline. Two major parameters have a pronounced impact on the degradation route and formation of 1-methylpyridinium, namely, the temperature and the nature of the trigonelline salt.

The failure to detect 1-methylpyridinium in previous model system studies (*4*, *8*) may in fact be due to employment of trigonelline hydrate, which shows a degradation pattern that differs from the hydrogen chloride salt. A familiar reaction of 1-methylpyridiniums is ring opening by reaction with nucleophiles, such as hydroxide ions, primary amines, and carbanions (*13*). The more basic hydrate may be present in the pseudobase form as 2-hydroxy-1-methyl-1,2-dihydropyridine, the open-chain tautomer (**Scheme 1**, route A), which could undergo hydrolysis to afford degradation products such as short-chain aldehydes and methylamine (*8*, *9*). In contrast, the replacement of the hydrate with hydrochloride or hydrogen sulfate counterions appears to confer resistance to the pyridine nucleus, possibly by electronically stabilizing the quaternary amine up to certain temperatures to afford 1-methylpyridinium as a major reaction product.

The formation of alkylpyridiniums such as 1,2-, 1,3-, and/or 1,4-dimethylpyridinium in this model system suggests that 1-methylpyridinium per se may further react with nucleophiles present in the reaction mixture, e.g., substituted pyridines. Thermally induced methyl rearrangements of quaternary salts such as 1-methylpyridine can procure a range of volatile products in the presence of a nucleophile such as pyridine, in particular good yields of 2- and 4-methylpyridine (30.1 and 34.6%, respectively), and minor formation of 3-methylpyridine (5.8%) and ethylpyridines (1.4%) (*14*, *15*) (**Scheme 1**, route B). Alkylpyridines thus act as nucleophiles and participate in methyl displacement reactions with 1-methylpyridinium (**Scheme 1**, route C) to afford pyridine and the new disubstituted

dialkypyridinium products (*9*). The latter may in principle react even further in a reverse "demethylation" reaction with available nucleophiles. In particular, the 2-substituted 1-methylpyridiniums show greater rate constants of demethylation, due to the impact of steric effects (*16*).

Depending on the nature of the salt, the various reactions described above may be driven to more defined "homogeneous" products as in the case of the hydrochloride or, as observed for trigonelline hydrate, toward pyridine ring-opening reactions that yield more or less undefined polymeric material that is difficult to isolate and characterize (**Scheme 1**, route A).

The second major thermal degradation route of trigonelline involves N-demethylation to provide nicotinic acid, the formation of which is positively correlated to the roasting degree of coffee (*7*) and corroborates the model described here. Nicotinic acid can be generated by hydrolysis of methyl nicotinoate (*8*) and then remains as a stable reaction product, resisting degradation to pyridine even at temperatures of ~230 °C (*6*). Interestingly, a significant impact of the salt form of trigonelline on the formation of the acid was also observed in this study, that is, far lower yields with trigonelline hydrate or hydrogen sulfate than with the hydrochloride. This indicates that methyl migration may be favored by chloride as the counterion, with a better yield of the progenitor methyl nicotinoate. Considering that trigonelline, pK = 2.92 (*17*), is present in its zwitterionic form, then the low yield of nicotinic acid in trigonelline hydrogen sulfate suggests rapid decarboxylation to afford a zwitterionic carbanion intermediate (*18*), which may hydrolyze or yield substituted alkylpyridines (*8*).

A number of model pyrolysis experiments have been described recently on trigonelline to investigate its potential mutagenic (*19*) or antimutagenic (*20*) effects in bacterial mutation assays. The results of such in vitro tests are in general difficult to interpret and extrapolate to in vivo conditions, which has been highlighted (*21*). Hence, some of these pyrolytic model studies may not be comparable because the nature of the salt of trigonelline is not documented or considered of importance. However, as shown in this work, the counterion plays a major role in defining the reaction route under mild pyrolytic conditions. Thus, an explanation for the contradictory findings of anti- and promutagenic activities of the trigonelline pyrolysis products may in fact be due to the usage of different salt forms of the quaternary amine and thus a significantly different product/activity profile.

At this stage, it is important to address the relevance of these model studies in the food product, that is, coffee. Depending on the nature of the trigonelline salt in the coffee bean, the degradation route may indeed favor the formation of 1-methylpyridinium, which will undoubtedly have a direct and indirect impact on other physicochemical properties such as flavor and aroma. In relation to the former, trigonelline per se is not a dominant contributor to roasted coffee bitterness (*22*), and alkylpyridiniums may indeed be responsible for a part of perceived bitterness in the brew. Furthermore, this compound was recently mentioned as a constituent of roasted coffee (*23*), but this observation is based only on global proton NMR investigations of the whole coffee brew, and no attempts were made to isolate and characterize the compound.

Investigations into the impact of coffee roasting on 1-methylpyridinium and dialkylpyridinium formation have recently been completed and are the subject of a following paper (*24*).

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LITERATURE CITED

(1) Bonham, C. C.; Wood, K. V.; Yang, W.-J.; Nadolska-Orczyk, A.; Samaras, Y.; Gage, D. A.; Poupart, J.; Burnet, M.; Hanson, A. D.; Rhodes, D. Identification of quaternary ammonium and tertiary sulfonium compounds by plasma desorption mass spectrometry. *J. Mass Spectrom.* **1995**, *30*, 1187−1194.

(2) Taguchi, H. Biosynthesis and metabolism of trigonelline, and physiological action of the compound. *Vitamins* **1988**, *62*, 549−557.

(3) Stennert, A.; Maier, H. G. Trigonelline in coffee. *Z. Lebensm. Unters. Forsch.* **1994**, *199*, 198−200.

(4) Viani, R.; Horman, I. Thermal behavior of trigonelline. *J. Food Sci.* **1974**, *39*, 1216−1217.

(5) Taguchi, H.; Sakaguchi, M.; Shimabayashi, Y. Trigonelline content in coffee beans and the thermal conversion of trigonelline into nicotinic acid during the roasting of coffee beans. *Agric. Biol. Chem.* **1985**, *49*, 3467−3471.

(6) Hughes, E. B.; Smith, R. F. The nicotinic acid content of coffee. *J. Soc. Chem. Ind.* **1946**, *65*, 284−286.

(7) Teply, L. J.; Prier, R. F. Nutritional evaluation of coffee including niacin bioassay. *J. Agric. Food Chem.* **1957**, *5*, 375−377.

(8) Rizzi, G. P.; Sanders, R. A. Mechanism of pyridine formation from trigonelline under coffee roasting conditions. In *Flavour Science, Recent Developments*; Taylor, A. J., Mottram, D. S., Eds.; Royal Society of Chemistry: Cambridge, U.K., 1996.

(9) Kosower, E. M. Additions to pyridinium rings. I. 1-Methylpyridinium iodide. *J. Am. Chem. Soc.* **1955**, *77*, 3883−3885.

(10) Hau, J.; Stadler, R.; Jenny, T.; Fay, L. B. Tandem mass spectrometric accurate mass performance of time-of-flight and Fourier transform ion cyclotron resonance mass spectrometry: a case study with pyridine derivatives. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1840−1848.

(11) Katritzky, A. R.; Banerji, A.; El-Osta, B. S.; Parker, I. R.; Ramsden, C. A. *J. Chem. Soc., Perkin Trans. 2* **1979**, *5*, 690−693.

(12) Casal, S.; Oliveira, M. B.; Ferreira, M. A. HPLC/diode-array applied to the thermal degradation of trigonelline, nicotinic acid and caffeine in coffee. *Food Chem.* **2000**, *68*, 481−485.

(13) Barton, D.; Ollis, W. D. *Comprehensive Organic Chemistry. The Synthesis and Reactions of Organic Compounds. Vol. 4. Heterocyclic Compounds*; Sammes, P. G., Ed.; Permagon Press: Oxford, U.K., 1979.

(14) Chumakov, Y. I.; Novikova, V. F. Alkylation and aralkylation of pyridine during the thermal decomposition of quaternary pyridinium salts by the Ladenburg-Chichibabin method. *Chem. Abstr.* **1968**, *69*, 43743m.

(15) Digiovanna, C. V.; Cislak, P. J.; Cislak, G. N. Some aspects of the direct alkylation of pyridine and methyl pyridines. *ACS Symp. Ser.* **1977**, *No. 55*, 397−407.

(16) Berg, U.; Gallo, R.; Metzger, J. Demethylations of quaternary pyridinium salts by a soft nucleophile, triphenylphosphine. Electronic and steric accelerations. *J. Org. Chem.* **1976**, *41*, 2621−2624.

(17) Niazi, M. S. K.; Mollin, J. Dossociation constants of some amino acid and pyridinecarboxylic acids in ethanol-H_2O mixtures. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 2605−2610.

(18) March, J. *Advanced Organic Chemistry. Reactions, Mechanisms, and Structure*, 4th ed.; Wiley: New York, 1992; p 564.

(19) Wu, X.; Skog, K.; Jägerstad, M. Trigonelline, a naturally occurring constituent of green coffee beans behind the mutagenic activity of roasted coffee? *Mutat. Res.* **1997**, *391*, 171−177.

(20) Nguyen, V. C.; Araki, Y.; Jinnouchi, T.; Murai, H. On the desmutagenicity of coffee. *Proceedings of the 16th International Colloquium on Coffee*, Kyoto, Japn; 1995; pp 88−93.

(21) Stadler, R. H.; Turesky, R. J.; Müller, O.; Markovic, J.; Leong-Morgenthaler, P.-M. The inhibitory effects of coffee on radical-mediated oxidation and mutagenicity. *Mut. Res.* **1994**, *308*, 177−190.

(22) Belitz, H. D. Geschmacksaktive Substanzen in Kaffee. *Proceedings of the 7th International Scientific Colloquium on Coffee*, Hamburg, Germany; 1975; Association Scientifique International du Café: Bremen, Germany, 1976; pp 242−252.

(23) Bosco, M.; Toffanin, R.; de Palo, D.; Zatti, L.; Segre, A. High-resolution ^{1}H NMR investigation of coffee. *J. Sci. Food Agric*. **1999**, *79*, 869−878.

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

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