Ammoniated Forage Poisoning: Isolation and Characterization of Alkyl-Substituted Imidazoles in Ammoniated Forage and in Milk

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A study has been performed to isolate and identify toxic nitrogen-containing compounds in ammoniated forages and in milk samples from sheep and a cow fed with ammoniated forages. Together with two previously identified imidazoles, 4-methylimidazole and 2-methylimidazole, the following imidazoles were isolated: 1,2-dimethyl-, 1,4-dimethyl-, 1,5-dimethyl-, 2,4-dimethyl-, and 2-ethyl-4-methylimidazole. The imidazoles were identified with gas chromatography/mass spectrometry and gas chromatography with nitrogen−phosphorus detector on a polar capillary column (Carbowax 51) after cleanup on a strong cation-exchange column.

Keywords: *Alkyl-substituted imidazoles; dimethylimidazoles; 4-methylimidazole; ammoniated forage poisoning; identification; GC/MS*

INTRODUCTION

Feeding ammoniated forage or ammoniated molasses may cause acute toxicity in ruminants, with violent symptoms of central nervous system (CNS) excitation (Bartlett and Broster, 1958; Morgan and Edwards, 1986a ; Brazil et al., 1994). The syndrome is commonly referred to as "bovine bonkers" or "crazy cow syndrome". A number of byproducts have been identifed after interaction of ammonia with reducing sugars in molasses (Wiggins and Wise, 1955; Nishie et al., 1969) and hay (Ray et al., 1984), of which the imidazoles and pyrazines seem to be the dominant groups. Nishie et al. (1969, 1970) tested the convulsant and lethal effects of imidazole, 1-methyl-, 2-methyl-, and 4-methylimidazole, and a range of pyrazines. These investigations indicated 4-methylimidazole (4-MeI) as the most probable compound responsible for the toxicity of ammoniated feeds. However, further studies by other authors have led to the conclusion that 4-MeI alone cannot explain the toxicity of ammoniated forages and that similar, but yet unknown, compounds may be involved (Morgan and Edwards, 1986b; Nielsen et al., 1986, 1993). A previous study performed by our department strongly supported this hypothesis. Quantitative determination of 4-MeI in samples of ewe milk at the time of poisoning and of plasma from poisoned lambs showed very low levels of 4-MeI, confirming the need to look for additional etiological agents (Sivertsen et al., 1993).

The formation of imidazoles and other heterocyclic compounds has been studied in different experimental ammoniated sugar systems, starting with the ammoniated molasses system of Wiggins and Wise (1955). Yamaguchi et al. (1979) identified thiophenes, pyrazines, pyrroles, furans, and imidazoles as principal constitutents in a heating model system of L-rhamnose/H_2S/NH_3. As a consequence of the assumption that 4-MeI is the main toxic compound in ammoniated sugar systems, 4-MeI concentrations have been studied in materials such as caramel (Cerny and Blumenthal, 1979), ammoniated forage (Ray et al., 1984; Karangwa et al., 1990), and biological material (Karangwa et al., 1990; Nielsen et al., 1993). Different techniques have been used for isolation and cleanup, including solvent extraction (Cerny and Blumenthal, 1979; Ray et al., 1984), ion exchange (Carnevale, 1975), and ion-pair extraction (Thomsen and Willumsen, 1981; Nielsen et al., 1993). The imidazoles have been identified and quantified by ion-pair liquid chromatography (Thomsen and Willumsen, 1981; Nielsen et al., 1993), GC analysis without derivatization (Cerny and Blumenthal, 1979), GC analysis after *N*-acetyl derivatization (Fuchs and Sundell, 1975), GC with nitrogen specific detector (Cerny and Blumenthal, 1979), and GC/MS (Ray et al., 1984; Fuchs and Sundell, 1975).

The purpose of the present study was to isolate and identify additional nitrogen-containing compounds that may be responsible for the intoxication of ruminants fed ammoniated forage. The samples used were both ammoniated forage and milk from animals fed with ammoniated forages.

MATERIALS AND METHODS

Milk and Forage Samples. Milk samples used were partly from a previously published intoxication study in sheep (Sivertsen et al., 1993) and partly from a cow specifically fed to obtain suitable material for the present investigation. The sheep milk samples were obtained by hand milking of two of the ewes in the experiment, at the time when the lambs sucking these ewes developed toxic symptoms. Blank samples were taken from the same ewes after they had changed to normal hay. The cow was of the standard Norwegian Red Cattle breed and was fed specially prepared ammoniated timothy hay from the day of calving onward. The milk from

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2 days postcalving was found to have the highest level of 4-MeI and was selected as main material for the study of cow milk. Milk from the same cow, produced 2 weeks later on regular timothy hay feeding, was used as blank sample.

The ammoniated forage samples were taken from the feed of the ewes and the cow, respectively, at the time the milk samples were collected. The sheep forage was from a bale of ammoniated timothy seed hay with high levels of 4-MeI, and the forage used for feeding was taken exclusively from the part of the bale lying on top during NH_3 treatment (Sivertsen et al., 1993; Randby and Langseth, 1990). The cow's forage was from the upper part of a bale of ammoniated regular timothy hay, otherwise produced and prepared in the same way as the ammoniated seed hay for the sheep. The use of regular hay for ammoniation in the cow experiment was done to achieve higher levels of sugars available for reaction with NH_3 during ammoniation.

Reagents. All chemicals were of analytical or HPLC grade. 1-Methyl-, 2-methyl-, 4-methyl-, 1,2-dimethyl-, 2,4-dimethyl-, and 2-ethyl-4-methylimidazole standards were obtained from Aldrich. 1,4-Dimethyl- and 1,5-dimethylimidazole were synthesized according to a standard method (Takeuchi, 1978) in the Department of Chemistry, University of Oslo.

Stock solutions of alkyl-substituted imidazoles (1 μg/μL) were prepared in acetonitrile. Standard solutions of 2, 5, 10, 20, and 40 ng/μL were used for spiking experiments.

Sample Preparation before Cleanup. Milk, 200 mL or available volume, was centrifuged for 15 min at 10 000 rpm and 4 °C. The aqueous fraction was decanted from the fat into a 1 L separatory funnel, and 200 mL of acetone was added. The mixture was then shaken for 4 min and centrifuged for 15 min. The supernatant was transferred to a 2 L round-bottom flask. The precipitated proteins were washed with an additional amount of acetone, which was combined with the first supernatant. Thereafter, the acetone was removed by careful evaporation at 40 °C. The remaining phase was adjusted to pH 2.3, and water was added to give the original volume of the sample, normally 200 mL.

Two grams of dried ground forage sample was weighed into a 150-mL Bühler homogenizing flask and 60 mL of 0.1 M HCl added. The sample was homogenized for 5 min and 40 mL of 0.1 M HCl added. The sample was centrifuged for 15 min. The pH of the supernatant was adjusted to 2.3 before cleanup on the cation-exchange column.

Cleanup Procedure. Pretreated samples of milk and forage were cleaned up on a strong cation-exchange resin. The cation-exchange resin was solvated with 50 mL of methanol and then pre-equilibrated with 50 mL of 0.005 M HCl before the extract was transferred to the column. The column was washed with 100 mL of 1 M acetic acid and then eluted with 80 mL of 3.8 M ammonia solution.

The eluate was adjusted to approximately pH 10 with 4 M HCl and extracted three times for 4 min with 150 mL of chloroform/ethanol (80 + 20). The sample was centrifuged if an emulsion appeared during the extraction. The combined chloroform/ethanol extract was dried with Na_2SO_4 for 30 min and thereafter concentrated to ≈3 mL using a rotary vacuum evaporator at 40 °C. The sample was not allowed to evaporate to dryness. The chloroform/ethanol concentrate was transferred quantitatively to a conical centrifuge tube and evaporated carefully on a heating block (40 °C) under a stream of nitrogen to a volume of ≈100 μL. The residue was then redissolved in 250 μL of acetonitrile, sonicated for 10 min, and mixed on a Whirlmixer (Fisons, Loughborough, U.K.) for 1 min. Finally, the sample was filtered through a Costar (Corning Costar Corp., Cambridge, MA) spin-X centrifuge filter unit with a 0.22 μm nylon membrane for 3 min.

Gas Chromatography (GC). All samples were analyzed by GC, performed on a Hewlett-Packard 5790 A series gas chromatograph equipped with a nitrogen−phosphorus detector (NPD) and a splitless injector. The column was a fused-silica capillary CP-Wax 51 (Chrompack, Middelburg, The Netherlands) column, 25 m × 0.25 mm i.d. (made for amines) with a film thickness of 0.20 μm. The carrier gas was helium. The temperatures of the injector and detector were 260 and 300 °C, respectively. The temperature program of the oven was as follows: 40 °C (2 min), 10 °C/min to 170 °C, 3 °C/min to 260 °C (15 min). A 1-μL sample was injected.

GC/Mass Spectrometry (GC/MS). The samples were analyzed by GC/MS using a Hewlett-Packard 5970 MSD and a Fison MD-800 mass spectrometer. The instruments were equipped with an electron impact (EI) ion source, and the spectra were recorded from *m*/*z* 40 to 250. The column, carrier gas, and temperature program used were identical with those for GC analyses. The amount injected was 2 μL.

High-Performance Liquid Chromatography (HPLC). Analyses were performed on a Perkin-Elmer HPLC system consisting of an LC 250 binary pump, an ISS 200 autosampler, and an LC 90 UV spectrophotometer. The signals were recorded on a Varian Star workstation. The injection volume was 20 μL. 4-MeI was analyzed according to a modified version (Sivertsen et al., 1993) of a method described by Thomsen and Willumsen (1981).

RESULTS AND DISCUSSION

Analytical Method. In contrast to previously published methods especially aimed at 4-MeI analysis, our analytical approach was designed to include all compounds that might contribute to the toxicity of ammoniated forage. The following assumptions were drawn. First, as these compounds obviously are produced during ammoniation of the forage and are absorbed by the animals efficiently enough to produce toxicity, they were assumed to be low molecular weight organic compounds with at least one nitrogen atom, which most likely means that they are basic compounds. The toxicity of milk to the sucklings (Weiss et al., 1986; Sivertsen et al., 1993) implies that the compounds are readily transferred to and most probably concentrated in milk. This indicates either a strong lipophilic character, which is unlikely in low molecular weight nitrogen-containing compounds, or a basic character, because of the lower pH value of milk compared to plasma. Thus, the method applied was designed to isolate relatively low molecular weight nitrogen-containing basic compounds from milk. Relatively large quantities of samples were used for sample preparation to detect compounds present in low concentrations. The ewe milk used was chosen because it had produced intoxication of suckling lambs. The milk from the ammoniated hay-fed cow was deliberately taken the first days postcalving, because we were aware that 4-MeI—and possibly other compounds of interest—might pass into colostrum in larger quantities than into normal milk (Motoi et al., 1997).

Imidazoles and other relatively polar compounds are not easily extracted into most organic solvents (Thomsen and Willumsen, 1981). 4-MeI can be analyzed by ion-pair extraction, giving recoveries of about 80 and 88% in milk and forage, respectively (Sivertsen et al., 1993). The optimum pH for this extraction is ∼6. However, weak bases do not form ion pairs at this pH. Therefore, a strong cation-exchange sorbent (SCX) was applied for cleanup of the different types of material in the present study.

The fat and the proteins had to be removed from the milk sample before it could be added to the cation-exchange sorbent to avoid clogging of the column. The fat formed a layer on the top of the sample after centrifugation at low temperature (4 °C). The proteins were precipitated by adding acetone. The acetone also ensured that the compounds of interest were released from the proteins and other macromolecules. The pH of the extract was adjusted to as low as 2.3 before it was applied to the cation-exchange sorbent to make sure

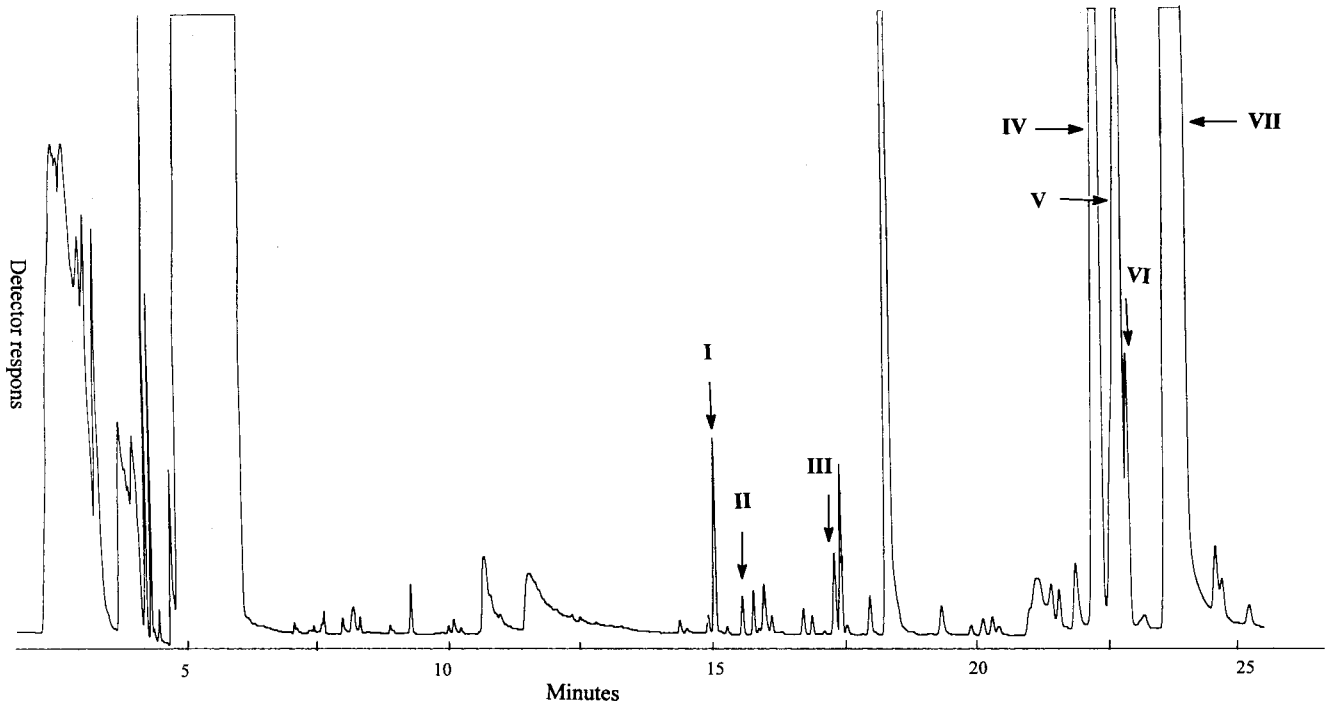

Figure 1. Gas chromatogram (NPD detector) of an extract of cow's milk. Identified alkylimidazole peaks: 1,4-dimethyl- (I), 1,2-dimethyl- (II), 1,5-dimethyl- (III), 2-methyl- (IV), 2,4-dimethyl- (V), 2-ethyl-4-methyl- (VI), and 4-methylimidazole (VII).

that the organic bases were positively charged. However, the recovery of very weak bases may have been limited because of incomplete ionization. To check the capacity of the column, sample volumes of 50, 100, 150, and 200 mL of pretreated cow milk were analyzed. The recovery of 4-MeI was just as good for a sample of 200 mL as for a sample of 50 mL of milk. A sample volume of 200 mL of milk was therefore used in this study.

The samples were not evaporated to dryness at any point in the procedure. When imidazoles and especially primary 1-substituded imidazoles were evaporated to dryness under a stream of nitrogen or in a freeze-dryer, the loss of these compounds was substantial. The boiling points of 1-substituted imidazoles are much lower than for the 1-unsubstituted imidazoles because hydrogen bonding is no longer possible when the nitrogen (N 1) is substituted (Gilchrist, 1992).

Another cleanup method, based on extraction of the sample with chloroform/ethanol, was also studied. The compounds were then further extracted into 0.1 M phosphoric acid and re-extracted into chloroform/ethanol. Data obtained by GC/MS showed that there was good agreement between the two methods, with similar recoveries for all of the identified imidazoles. However, there were fewer interfering compounds from the sample matrix in the extracts from the strong cation-exchange sorbent.

The samples were analyzed by GC on a Carbowax 51 capillary column, which is a polar column especially made for amines. The imidazoles could then be chromatographed without being derivatized. By using an NPD both high sensitivity and selectivity for nitrogen-containing compounds were obtained. The samples were also analyzed by GC/MS with the same column. The compounds were identified by comparing retention times and MS spectra with those of corresponding standards. Compounds also found in the blank milk samples were excluded from further consideration.

The extracts were also analyzed by HPLC with reverse-phase ion-pair chromatography. 2-Methyl- and 4-methylimidazole were well separated. 2,4-Dimethyl- and 1,2-dimethylimidazole eluted together, as was also the case for 1,4-dimethyl- and 1,5-dimethylimidazole. Substituted imidazoles were better separated by gas chromatography than by HPLC. Despite the relatively low volatility and high polarity of these compounds, GC was found to be the most rewarding technique in this study, although 4-MeI had to be analyzed by reverse-phase ion-pair chromatography on HPLC.

Identification of New Alkylimidazoles. The extracts of milk from sheep and a cow fed ammoniated forage were primarily found to contain alkyl-substituted imidazoles. The following substituted imidazoles were identified in forage and milk from sheep and cow: 2-methyl-, 4-methyl-, 1,2-dimethyl-, 1,4-dimethyl-, 1,5-dimethyl-, 2,4-dimethyl-, and 2-ethyl-4-methylimidazole. None of the dialkylimidazoles have been identified in ammoniated forage before. Yamaguchi et al. (1979) identified 2-methyl-, 2,5-dimethyl-, 2-ethyl-, and 2-ethyl-5-methylimidazole in a mixture of L-rhamnose/H_2S/NH_3. The two pairs 2,4-dimethyl- and 2,5-dimethylimidazole and 2-ethyl-4-methyl- and 2-ethyl-5-methylimidazole are both proton tautomers (Gilchrist, 1992). In Figure 1 is shown a gas chromatogram with NPD detection of an extract of milk from the cow fed ammoniated forage. The Carbowax 51 capillary column afforded complete separation of the different imidazoles but not between 4-MeI and an unknown compound also present in the blank milk samples. The separation of 4-MeI and this unknown matrix compound was, however, sufficient to obtain pure mass spectra. Imidazoles that contain a free NH group elute later than 1-substituted imidazoles (Ray et al., 1984).

The mass spectra of the identified imidazoles are shown in Figure 2. The molecular ion peaks and the fragmentation patterns indicate that these compounds contain an imidazole skeleton with one or more alkyl substitutents, methyl, ethyl, or propyl. Dominant peaks in the mass spectra of alkyl-substituted imidazoles include M − 1, *m/z* 54 or 68 (M − 1 − R_2CN), and 42

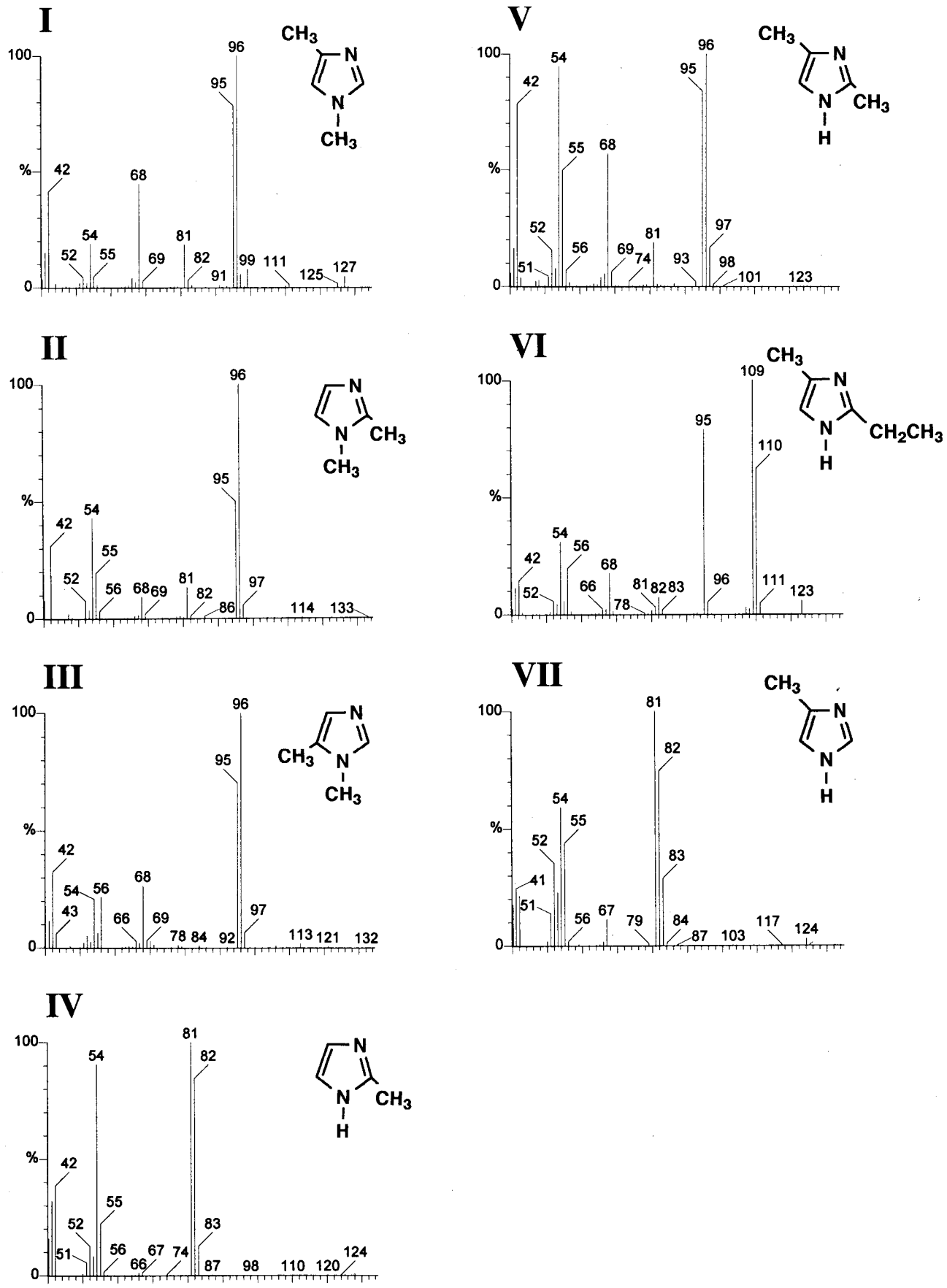

Figure 2. Mass spectra and molecular structures of identified alkylimidazoles: 1,4-dimethyl- (I), 1,2-dimethyl- (II), 1,5-dimethyl- (III), 2-methyl- (IV), 2,4-dimethyl- (V), 2-ethyl-4-methyl- (VI), and 4-methylimidazole (VII).

(see Figure 2). Loss of an H-radical from one of the alkyl substituents R_1, R_2, or R_4 gave rise to m/z M − 1. Further fragmentation of alkyl-substituted imidazole is proposed: The imidazole ring is opened by breaking the

bond between N-1 and C-2. Cleavage of the N-3−C-4 bond and loss of R_2CN gave rise to *m/z* 54 or 68 depending on the mass of the alkyl substituent R_1, R_2, or R_4. Further decomposition of the fragment *m/z* 54 or 68 from 1,2-dimethyl, 1,4-dimethyl-, or 1,5-dimethylimidazole gave rise to *m/z* 42 or 28 (not shown in the mass spectra). However, the differences between mass spectra of structural isomers of alkylimidazoles are limited. Comparisons of retention times with pure standards were therefore necessary to obtain accurate identification of the imidazoles. Both spectra and the retention times of the compounds were in good accordance with the authentic standards.

Pure standards were not available for some of the compounds detected. These compounds showed EI spectra with fragmentation patterns similar to those of the alkyl-substituted imidazoles and pyrazoles (McLafferty and Stauffer, 1989; Figure 3). An unidentified compound **1**, found in cow milk and sheep forage, eluted after 13.3 min and gave a molecular ion peak at *m/z* 110 (Figure 3). The short retention time indicates that compound **1** could be a 1-substituted imidazole or a pyrazole. The intensity of the peak *m/z* 95 (M − 15) was low and indicates that the molecule does not contain an ethyl group. On the basis of these observations, we propose that the structure of compound **1** is 1,2,4-trimethylimidazole. The mass spectrum of a compound **2** found in cow milk (RT = 18.1 min) showed two major peaks, *m/z* 124 (M^+) and *m/z* 109 (M − 15) and no peak at *m/z* 95(M − 29). These observations indicated that compound **2** does not contain a propyl group. We propose that the compound is a diethyl or ethyl, dimethyl-substituted imidazole or pyrazole. The mass spectrum of an unknown compound **3** (forage to sheep, RT = 20.6) contained a molecular ion at *m/z* 96. The intensity of the peak *m/z* 81, which corresponds to a loss of methyl group, was low. This indicated that compound **3** does not contain any ethyl substituent. We propose that the structure is a dimethyl-substituted imidazole or pyrazole.

Pyrazine and 2-methyl-, 2,3-dimethyl-, 2,5-dimethyl-, and 2,6-dimethylpyrazine were also identified in forage and cow milk, confirmed by comparison with authentic standards using GC/MS. The concentrations were, however, substantially lower than for the alkylimidazoles.

The HPLC chromatograms verified that the extracts mainly contained the identified alkylimidazoles. Other unobserved compounds may as well have been present in our extracts, but only in very low concentrations. The peak described by Weiss et al. (1986) was detected using the same cleanup and HPLC procedure as described by those authors. The peak was detected with a fluorescence detector, but we found no or only a very small sign of it in UV, indicating that the substance was present only in very small amounts in our material. Due to the instability observed during evaporation, further studies of that compound by GC/MS were not possible.

Toxicological Aspects. The new dialkylimidazoles identified may add to the toxicity of ammoniated forage. However, we have not found them to be more toxic than 4-MeI in mice, and their concentrations in ammoniated forage and in milk were found to be from 30% less to 2 orders of magnitude lower than the concentration of 4-MeI (Müller et al., 1998). Therefore, the identification of these new dialkylimidazoles does not solve all of the riddles of ammoniated forage poisoning.

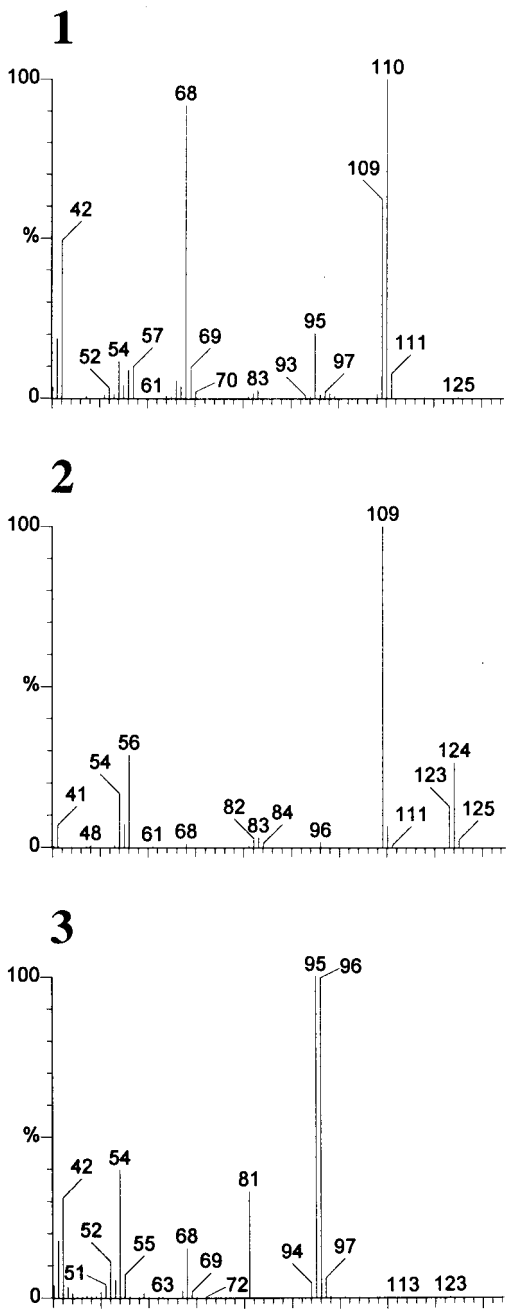

Figure 3. Mass spectra of unidentified imidazoles or pyrazoles: MW 110 (1), MW 124 (2), MW 96 (3).

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