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Fast Determination of Histamine in Cheese by Nuclear Magnetic Resonance (NMR)

E. SCHIEVANO,* K. GUARDINI, AND S. MAMMI

Department of Chemical Sciences, University of Padova and Institute of Biomolecular Chemistry, National Research Council (CNR), Via Marzolo 1, Padova 35131, Italy

A rapid and quantitative ^{1}H nuclear magnetic resonance (NMR) method was developed to analyze histamine in cheeses. The procedure is simple because the acid extract is analyzed directly, without any need for further filtration, derivatization, or other manipulation. This NMR method was demonstrated to be specific by 2D total correlation spectroscopy (TOCSY) and heteronuclear multiple-quantum coherence (HMQC) experiments and reliable in terms of linearity, accuracy, recovery, repeatability, and limits of detection (LOD). Good precision, with relative standard deviation (RSD) < 4%, recovery of 100%, and a range of 0.6−1 mg/kg for the LOD were obtained. The NMR method was successfully applied to different types of cheese, ranging from soft to hard. No interference from free amino acids, proteins, and other natural components was detected. The NMR method could be transferred to other kinds of food.

KEYWORDS: Histamine; quantitative NMR; food analysis

1. INTRODUCTION

Biogenic amines (BA) are nitrogen compounds that naturally occur in all foods that contain proteins or free amino acids. They derive mainly from microbial decarboxylation of amino acids and transamination of aldehydes and ketones (*1*). Small amounts of some BA can usually be found in some foods, because they play a natural role in microbial plant and animal metabolism.

Several types of fermented milks and cheeses are major sources of dietary BA (*2*), because of their high protein content (*3*). During cheese ripening, degradation of casein occurs, leading to the accumulation of free amino acids (*4*) that can be converted into BA by the activity of bacterial decarboxylases (*5*). Cheese, similar to other fermented foods, is an ideal substrate for amine production. The environmental conditions of its manufacturing process (optimal pH, temperature, salt, and water availability and the presence of suitable cofactors, such as pyridoxal phosphate) allow for the growth of decarboxylase-positive microorganisms and enzyme activity (*6*).

Some amines, such as tyramine, histamine, and serotonin, can have direct or indirect effects on the human vascular and nervous system. A large amount of BA can cause a rash, headache, nausea, hypo- or hypertension, cardiac palpitation, intracerebral hemorrhage, and anaphylactic shock (*2, 7, 8*). These effects can be especially adverse in patients treated with classical monoamine oxidase inhibitory drugs (*9*).

A strong hypertensive response (the "cheese reaction") can take place for histamine intakes exceeding 1000 mg/kg. The critical dose of oral histamine has been estimated to be in the range of 100−200 mg (*10*). Despite the fact that cheese may contain exceedingly high levels of histamine and other BA (>2000 mg/kg), tolerances have not been set thus far. Only for histamine in fish, a limit has been established by the European Union, the U.S. Food and Drug Administration (FDA), and several other countries, at 50−200 mg/kg of fish tissue (*11*−*13*).

The amine content in cheeses varies according to samples, from nondetermined (ND) to 4200 mg/kg (*14*−*16*). This wide variability may depend upon the type of cheese, the ripening time, the manufacturing process conditions, and the microorganisms present (*5*). The microorganisms involved in amine production may be the starter cultures used to control the raw milk flora or may be introduced by contamination before, during, or after cheese making and storage (*15, 17*). Thus, the amounts of BA can be a parameter of hygienic quality in cheese making (*18, 19*). In addition, they can serve as indicators of the authenticity of some particular cheeses (*20*).

Several analytical techniques have been proposed for the determination of BA in various foods (for a recent review, see ref *21*). Most are chromatographic methods and include capillary electrophoresis (CE), thin-layer chromatography (TLC), gas chromatography (GC), ion-exchange chromatography, and high-performance liquid chromatography (HPLC).

Among the cited techniques, reverse-phase (RP)-HPLC is considered the most suitable one. Several detection techniques, such as ultraviolet (UV), electrochemical, and fluorescence, have been applied (*21*). To ensure adequate sensitivity, a derivatization step is generally performed before injection.

Sample derivatization is usually costly; it requires extensive sample cleanup and has a low sample throughput. Dependent upon the complexity of the matrix and the amount of free amino acids that can compete with the derivatizing agent, a further

* To whom correspondence should be addressed. Telephone: 39-0498275742. Fax: 39-0498275239. E-mail: elisabetta.schievano@unipd.it.

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purification step may be necessary after extraction of the BA from the solid matrix in an acidic medium. Often, a second extraction with an organic solvent is used to selectively dissolve free amines, leaving the free amino acids in the aqueous layer. Because optimal extraction of different amines occurs at different pH values, a strict control of this parameter is needed to ensure satisfactory recoveries and reproducibility (*22*). Direct derivatization of the acidic extract was successfully used by a number of authors (*8, 18, 23*) for food matrices with relatively low free amino acid contents, such as meat, fish, and vegetables (*22*). Recently, it was also employed with cheese samples with high concentrations of free amino acids (*24*). In any case, each step reduces the recovery, increases the risk of contamination and accidental loss of analyte, and increases the time needed for the analysis.

Some analytical methods that do not require derivatization have appeared in the literature (*25*−*27*). Although they have the distinct advantage of a reduction in the analysis time, they do not achieve satisfactory recovery, selectivity, or trueness.

Cheese is a complex matrix principally because of its high proportion of fat, protein, peptides, and inorganic cations. Furthermore, cheese is a foodstuff that can change very much depending upon its origin, conditions of the manufacturing processing, microbial flora, enzyme activities, freshness, or ripening time. All of these parameters can affect the analytical efficiency, and for these reasons, the determination and detection of histamine is more difficult in cheese than in other matrices.

This work describes a new method for the quantitative determination of histamine in cheese based on nuclear magnetic resonance (NMR). A powerful characteristic of NMR spectroscopy is the possibility to identify each compound in a complex mixture simultaneously and nondestructively (*28*). Quantification of single constituents in mixtures is also possible through integration of the area of the NMR signal (*29*−*32*).

The method that we propose is rapid because the acid extract is analyzed directly, without any need for further filtration, derivatization, or other manipulation. Although demonstrated on several types of cheese, it could be applied to other kinds of food, such as yogurt, beer, fish, and ripened-fish products, as well as cooked and fermented sausages.

2. MATERIALS AND METHODS

2.1. Chemicals. All reagents were of analytical grade or of the highest grade available.

N,*N*-Dimethylformamide (DMF) (99.9%), histamine dihydrochloride (99%), and europium(III)-tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionate [Eu(fod)$_3$, 99%)] were purchased from Sigma-Aldrich. Deuterated solvents ($CDCl_3$, D_2O, and DMSO-d_6) (>98% D) were purchased from Eurisotop.

2.2. Preparation of Standard Solutions. Fresh histamine stock solutions were prepared every day by dissolving 3 mg, accurately weighed (±0.01 mg), in 1 mL of D_2O at about pH 1.7. The working solutions were obtained, diluting the histamine stock solution with the same solvent.

2.3. Samples. All samples were purchased from Italian stores. One sample of each of the following 10 different types of cheese was analyzed: Asiago, a bovine hard cheese; Asiago DOP, a ripened bovine hard cheese; Parmigiano Reggiano, a bovine hard cheese, aged 24 months; Montasio DOP, a bovine hard cheese, aged 12 months; Emmental, a bovine medium−hard cheese; Tilsit, a bovine semisoft cheese; Bergkase, a bovine semisoft cheese; Gorgonzola, a bovine soft cheese; Pecorino Grotta, an ovine semisoft cheese; Pecorino Pienza, a ripened ovine hard cheese.

2.4. Sample Preparation. Cheese samples were frozen in liquid nitrogen and then ground with a blender. An amount (0.5 g) of ground sample was weighed directly in a test tube and extracted with 2 mL of 0.25 M $HClO_4$ in D_2O, vortexing for 10 min. This $HClO_4$ concentration assures that the pH value in the final extract is always lower than 1.5. The pH was measured using a Metrohom pHmeter. The supernatant was ultrafiltered using a Vivaspin device (Sartorius) with a 2000 molecular-weight cutoff at 6500 rpm for 15 min at 4 °C to remove proteins and peptides. For all NMR spectra, 0.5 mL of extract was transferred into 5 mm precision glass NMR tubes (Wilmad 535-pp). Solutions were well-mixed and freed from bubbles.

2.5. NMR Spectroscopy. NMR experiments were carried out using a Bruker Avance DMX600 instrument, operating at 600.01 MHz for protons and equipped with a 5 mm TXI *xyz*-gradient reverse probe. Data were processed using the ACD software (ACD/Specmanager 7.00 sofware, Advanced Chemistry Development, Inc., Toronto, Ontario, Canada). Fourier transformation was performed after zero filling the free induction delay (FID) data to 131 072 points and after apodization using a decreasing exponential with line broadening of 0.2 Hz. Integral values were entered into Microsoft Excel spreadsheets for further processing and statistical comparisons.

The longitudinal relaxation time T_1 of all signals of interest, both in the standard solutions and in the matrices, was determined using the inversion−recovery sequence.

The 1D spectra were acquired using a double pulsed field gradient spin echo sequence incorporating an adiabatic inversion Gaussian pulse (*33*) of 2 kHz sweep width and 10 ms duration. All gradient pulses were followed by a 100 μs recovery delay. Rastrelli et al. (*33*) demonstrated that the excitation profile exhibits a flat region spanning 1500 Hz and a well-behaved phase, both of which are essential requirements for quantitative applications.

In all experiments, a spectral width of 6000 Hz, 32 768 data points, and a receiver gain of 16 384 were used. The flip angle and the number of scans changed depending upon the histamine concentration. Specifically, with a histamine content higher than 100 mg/kg, 32 scans and 30° flip angles were used. For samples with histamine concentration ranging from 100 to 30 mg/kg, the number of scans was increased to 64. In the case of histamine concentrations lower than 30 ppm, 90° pulses and 96 scans were used. To produce quantitative data, the relaxation delay was set based on the longest measured T_1. Specifically, it was at least $5T_1$ and $3T_1$ when 90° and 30° hard pulses were used, respectively.

Peak identification was confirmed through the addition of pure analyte and through 2D spectra. ^{1}H−^{1}H total correlation spectroscopy (TOCSY) experiments were recorded in the time-proportional phase increment (TPPI) mode, with a spectral window of 10 ppm in both dimensions, 2048 data points in F2 and 512 increments in F1, and water presaturation during the 2 s relaxation delay. A mixing time of 70 ms was chosen as the most suitable value. A heteronuclear multiple-quantum coherence (HMQC) experiment was performed with 64 scans, 200 × 1024 points data matrix, and a spectral width of 10 ppm in the ^{1}H dimension and 220 ppm in the ^{13}C dimension. To obtain a higher resolution in the ^{13}C dimension, a folded HMQC spectrum was acquired, in which a spectral width of 30 ppm centered at 125 ppm was used.

To quantify absolute concentrations, an external standard was used: a coaxial insert (wgs-5bl, Wilmad Glass), filled with a DMF solution in D_2O, was placed inside the NMR tube. The concentration of the standard was a compromise between a good S/N ratio and signal intensity, comparable to that of the test substance. The absolute concentration of histamine was determined by integration of the area of the aromatic proton H2 of the imidazole ring (I_a) and that of the aldehydic proton of DMF (I_s) at 7.92 ppm. The DMF concentration was determined gravimetrically.

The use of DMF as an external standard has several advantages: DMF can be purchased very pure, is soluble in the chosen solvent, is stable for a long time under the experimental conditions, and gives a singlet in a free region of the NMR spectra in all matrices studied (*29*).

To calculate the analyte concentration, the ratio between the volume of the coaxial insert (V_c) and the volume of the NMR tube (V_t) must be carefully determined (*34*). To this end, both the coaxial insert and the NMR tube were filled with proper aliquots of a 1 mM solution of acetone in acetone-d_6. A small amount of Eu(fod)$_3$ was added to the solution in the external tube to shift the acetone signal by approximately 0.1 ppm (60 Hz) downfield. Because the

inner and the external acetone concentrations are identical, the ratio of the two integrated signals gives the V_c/V_t ratio directly. In this way, a value of $V_c/V_t = 0.108$ was determined and used thereafter as the scale factor. The molar concentration of analyte in the NMR solution is given by C_a (mol/L) $= (I_a/I_s)(V_c/V_t)C_s$, where C_s is the concentration of the external standard (*35*).

An alternate approach would be to contain the external standard in a separate, identical 5 mm tube, tune and match the probe, and determine the 90° pulse for each sample (*35*).

After selecting the optimal conditions for data acquisition (i.e., correct instrumental parameters, such as flip angle, relaxation delay, field homogeneity, etc.), repeated runs with the same sample performed in different days (also several days later) showed perfectly overlapping spectra. Our results are in agreement with investigations on instrumental stability; an uncertainty of less than 1.5% was reported when changing the NMR probe and the operator and making a series of measurements over a period of 5 months (*36*). The greatest source of error is in the integration step: the main limitation of quantitative NMR (qNMR) is data processing, which heavily influences the integration values (*35*).

The integration accuracy was estimated by calculating the trueness and the precision of the peak area of selected resonances. Accurate results were obtained by extremely careful manual phase and baseline correction, as well as manual integration. The quantification of signals relies on the correct choice of the integration interval. The best result was obtained when each signal was manually integrated in each individual spectrum and when the integral ratios between the standard and the analyte signals were calculated using identical criteria to select the tails (*36*, *37*).

2.6. Quantitative Determination of Histamine. In conformity with the Eurachem guidelines (*38*), the proposed method was validated in terms of instrumental linearity, precision, trueness, analytical recovery, as well as limits of detection and quantitation. All of the parameters described below were determined considering three different preparations per cheese sample, one spectral acquisition per preparation, and six repetitions of the data processing per spectrum, for a total of 18 measurements per point.

2.6.1. Instrumental Linearity and Accuracy. These parameters were tested using five standard histamine solutions in the concentration range of 6−300 ppm. The NMR (detector) response was tested by regression analysis of the processed data. The precision of the method was determined by calculating the relative standard deviation (RSD, %) for the repeated measurements (18 measurements per point, as described before). The trueness of the method was expressed in terms of bias by assessing the agreement between the measured (mc) and nominal (nc) concentrations of the analyzed samples: (mc − nc) × 100/nc.

2.6.2. Analytical Recovery and Accuracy. Recovery was tested through the standard addition procedure on a bergkase cheese sample, in which no histamine was detected. The cheese was spiked before sample preparation at three different concentration levels of histamine, i.e., 300, 200, and 30 mg/kg.

2.6.3. Analytical Repeatability. Repeatability of the entire analytical procedure, in terms of RSD, was tested with each of the 10 different cheeses.

2.6.4. Limits of Detection and Quantitation. The limits of detection (LOD) and quantitation (LOQ) are defined as the analyte concentrations at which signal-to-noise ratios of at least 3:1 and 10:1 are obtained, respectively. These limits were determined using a cheese sample in which no histamine was detected, spiked with 2, 4, 6, 9, and 15 mg/kg of histamine.

3. RESULTS AND DISCUSSION

3.1. Spectral Analysis. In **Figure 1A**, the complete spectrum of a cheese extract acquired with a single-pulse sequence with presaturation of the water signal is reported. The spectrum shows strong signals in the aliphatic region and weaker signals in the aromatic region, where protons from histamine and histidine also fall. In the case of NMR, the ability to record the weaker signals relies essentially on the dynamic range of the analogue-to-digital converter (ADC): when most of this range is used to digitize the strongest signals, the weak ones may lie close or below detectability. The sequence used in this work (*33*) allowed us to eliminate all aliphatic signals and the water signal without the necessity to use water presaturation and to detect the signals of interest with a maximized value of the receiver gain. **Figure 1B** shows the results of one of these experiments, and expansions of the aromatic region are reported in **Figure 1C**. The resonances of the H2 protons of both histamine and histidine and that of the external standard are indicated. These protons (**Figure 1**) were chosen for quantitation because the H5 protons fall in a crowded region. On the other hand, the peak of the H2 proton of histamine is well-resolved from that of the corresponding histidine proton and from other signals. The spectral resolution of the histamine H2 signal depends upon the pH of the extract, as shown in **Figure 2A**. The maximum peak separation is reached at pH < 1.5. An acidic pH value is also optimal for our aim because it is known that, in these conditions, a quantitative extraction is obtained.

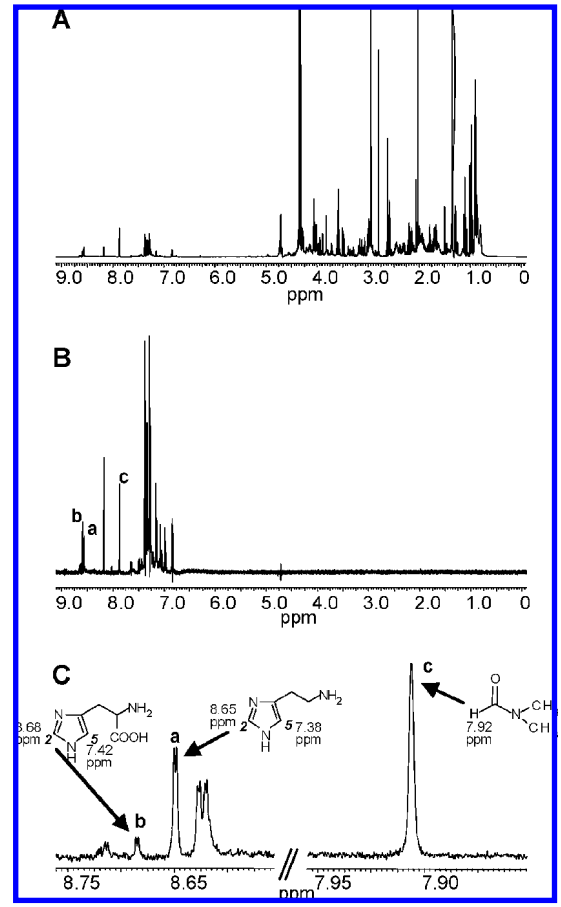

Figure 1. **(A)** Complete 1D spectrum of a cheese extract acquired with a single-pulse sequence and water presaturation. **(B)** Complete 1D spectra of the same cheese extract acquired with a double pulsed field gradient spin echo sequence incorporating an adiabatic inversion Gaussian pulse (*33*). **(C)** Expansion of the aromatic region of the spectrum of B: (a) histamine, (b) histidine, and (c) DMF.

Once the appropriate signal in the spectrum (H2) is identified, it is necessary to acquire the spectrum in a way that would ensure proportionality between signal intensity and the total number of nuclei contained in the NMR sample tube, for both the analyte and the standard compound. It is necessary to allow enough time for the magnetization to recover the equilibrium value before the next pulse cycle can begin. The length of the recycle delay is governed by the relaxation properties of the individual nuclei in the sample. To ensure almost quantita-

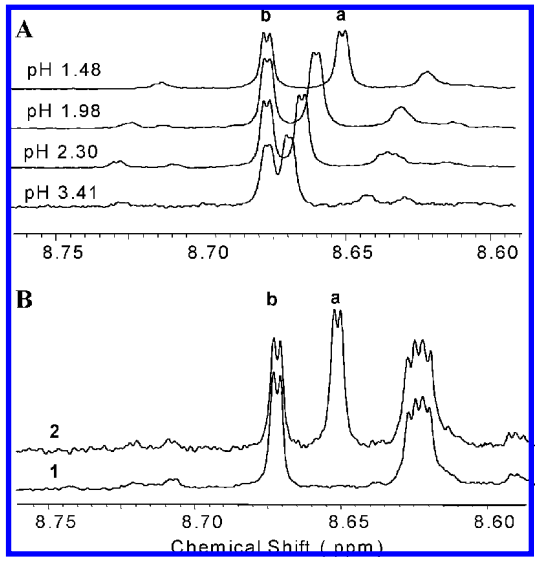

Figure 2. **(A)** Expanded regions of spectra of a cheese extract obtained at different pH values (indicated). Peaks from the H2 of (a) histamine and (b) histidine are labeled. **(B)** Portions of spectra of a sample in which no histamine was detected, (1) before and (2) after the addition of histamine.

tive recovery of the magnetization, $5T_1$, $4T_1$, and $3T_1$ must be employed when a flip angle of 90°, 50°, and 30° is applied, respectively. The chosen longitudinal relaxation time (T_1) is the longest among the signals of interest. In the present case, this signal is the H2 proton of histamine in both standard solutions and the matrices under study, with T_1 values of 15.1 and 10.6 s, respectively.

A key pre-requisite for qNMR investigations is to confirm that the signal of interest is only due to the analyte and not to other components. The identity was confirmed by adding amounts of standard analyte to the solution and by identifying which signal increased. Portions of spectra of a sample in which no histamine was detected before and after the addition of a histamine standard are reported in **Figure 2B**. In all of the samples checked (10 different types of cheese), the histamine resonance falls at exactly the same chemical shift (8.65 ± 0.02 ppm). We also tested for selectivity, i.e., the ability to determine analytes of interest in a complex mixture without interference from other components in the mixture (*38*). Because of the very different nature of the samples considered, in a few samples, partial overlap with another signal was observed. Small pH changes (±0.3 pH units) directly in the NMR tube always resolved the signals. To exclude exact signal overlap of other compounds with that of the analyte, which would not be observed in a 1D spectrum, we acquired 2D experiments to detect possible overlap in the second dimension. Portions of a representative TOCSY spectrum are reported in **Figure 3**. Only one cross-peak is present at the H2 resonance frequency, corresponding to the scalar coupling of H2 with H5. To exclude also the presence of overlapping singlets that do not give rise to cross-peaks in a TOCSY spectrum, two HMQC experiments were performed and are reported in **Figure 3**. Only one ^{13}C resonance is observed at the H2 frequency. These experiments were performed on two different cheese samples, obtaining the same results. We can conclude that the resonance at 8.65 ppm is specific for histamine.

3.2. Instrumental Linearity and Accuracy. The instrumental linearity was checked reporting the experimentally determined concentrations versus the gravimetric reference values obtained for standard histamine solutions. The regression showed good linearity. Least-squared analysis produced a correlation coefficient of 0.999 98 and a regression equation of $y = 1.0046x$. These results confirm a linear relationship between the amine concentration and instrumental response in the range of 0−300 mg/L, which corresponds to the range of 0−1200 mg/kg in the matrix.

The instrumental accuracy of the NMR measurements was tested performing three replicates and six repetitions of the data processing per spectrum. Mean (x), relative standard deviation (RSD), and bias, obtained at five different concentration levels, are reported in **Table 1**. The RSDs obtained were always satisfactory, and they were acceptable according to Horwitz's formula (RSDH) (*39*). Good trueness values were also obtained.

3.3. Extraction Optimization. Essential to the validation of the entire analytical procedure is to obtain quantitative histamine extraction. The correct sample mass/solvent volume ratio (m/v) to achieve this was optimized using a cheese containing an average amount of histamine (∼125 mg/kg). Three separate extractions per m/v ratio were performed. The data reported in **Table 2** demonstrate that exhaustive extraction is reached already at a 1:3 m/v ratio. The recovery determination (see below) demonstrates that the extraction is also quantitative. In any case, we chose the extraction m/v of 1:4, as described in the Materials and Methods, as a compromise between the need to ensure quantitation also at higher histamine levels and to maintain good LOD and LOQ levels.

3.4. Analytical Results. To evaluate the accuracy and the extraction efficiency of the proposed method, recovery experiments were performed on a sample in which no histamine was detected, spiked at three different concentrations of standard histamine. The results presented in **Table 3** are satisfactory. Recovery close to 100% was observed at all spiking levels.

The repeatability of the entire analytical procedure was determined using eight cheese samples. In the other two samples, the histamine concentration was lower than the LOD. The histamine concentration found in these samples ranges from 32 to 600 mg/kg. The RSD values range from about 4% at low histamine concentrations to about 2% at higher levels (**Table 4**). The values of RSD demonstrate the precision of the method.

3.5. LOD and LOQ. In NMR spectroscopy, the LOD and LOQ values depend upon acquisition parameters, mainly on the number of scans that influence the S/N ratio and the experimental time. The LOD and LOQ values were determined with two different numbers of scans (128 and 256, with identical parameters otherwise). With an acquisition time of 1 h and 20 min, a LOD of 1.09 mg/kg and a LOQ of 3.79 mg/kg were obtained, with a R^2 for the linear equation of 0.9958. With a longer acquisition time (3 h and 40 min), a LOD of 0.56 mg/kg and a LOQ of 2.71 mg/kg were found, with a R^2 of 0.9979. We can conclude that, even using the shorter acquisition time, the LOQ is adequately low.

A simple, rapid, and specific NMR method to quantify histamine in cheese samples was described. Samples of 10 different types of cheese, both hard and soft, were successfully tested. Free amino acids, peptides, or other compounds do not interfere with the determination. This method has the advantage of reducing the extraction time because it directly analyses the acid extract without the necessity to separate or derivatize histamine or to change the pH, steps that are usually considered critical.

Extraction of histamine from cheese is quantitative. The accuracy of the NMR method in terms of precision and trueness

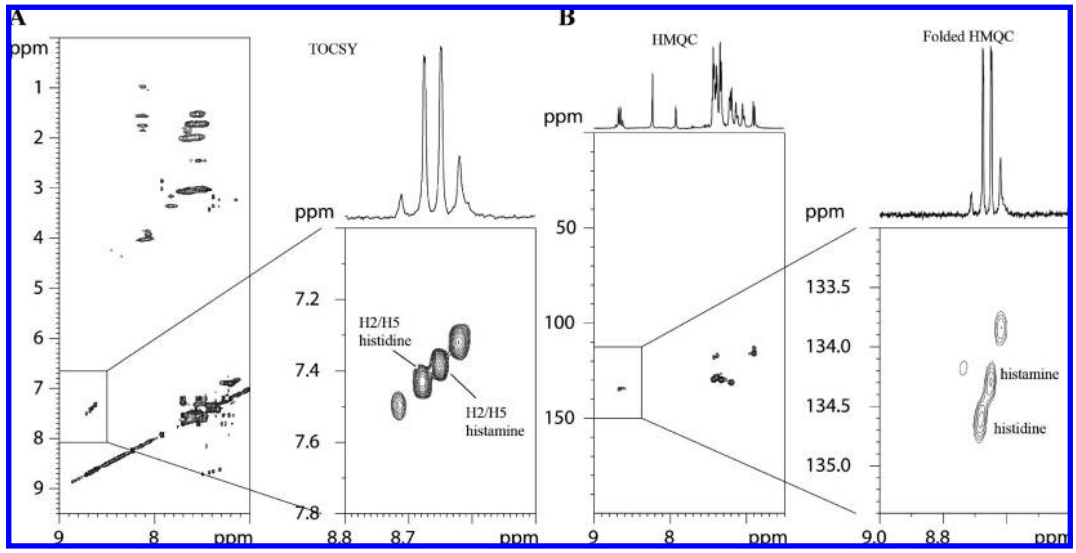

Figure 3. **(A)** Portion of a TOCSY spectrum of a cheese extract and expansion of the region including the only cross-peak present at the histamine H2 resonance frequency. **(B)** Portions of an HMQC spectrum of a cheese extract and a folded HMQC spectrum including the only peak present at the histamine H2 resonance frequency.

Table 1. Instrumental Accuracy

histamine concentration[a] (mg/kg)	bias[b] (%)	RSD[c] (%)	RSDH[d] (%)
301.1 ± 0.8	0.5	1.2	6.8
164.0 ± 0.5	0.7	1.1	7.4
99.7 ± 0.5	−0.4	1.7	8.0
30.3 ± 0.1	−0.6	1.3	9.6
5.74 ± 0.07	0.44	4.2	12.3

[a] Mean of 18 measurements per point, obtained from three different preparations per histamine concentration level, with one spectral acquisition per preparation and six repetitions of the data processing per spectrum. [b] (mc − nc) × 100/nc, where mc is the measured concentration and nc is the nominal concentration. [c] Relative standard deviation. [d] Acceptable relative standard deviation according to Horwitz's formula for intralaboratory studies.

Table 2. Extraction Efficiency Test

ratio of m (g)/v (mL)[a]	histamine concentration (mg/kg)[b]	RSD (%)
1:2	104.5 ± 0.9	3.2
1:3	126.0 ± 0.5	1.7
1:4	124.7 ± 0.7	2.2
1:10	123.9 ± 0.8	2.5

[a] m/v = sample mass/solvent volume ratio. [b] Mean of 18 measurements per point, obtained from three different preparations per extraction, with one spectral acquisition per preparation and six repetitions of the data processing per spectrum.

Table 3. Recovery of Histamine and Accuracy of the Method in Spiked Samples

histamine concentration (mg/kg)[a]	% recovery (SD)	bias (%)	RSD (%)
35	100.8 (3.2)	1.0	2.4
220	98.7 (1.0)	−1.4	0.9
350	99.0 (0.9)	−0.99	0.8

[a] Mean of 18 measurements per point, obtained from three different preparations per histamine concentration level, spiking the samples before preparation, with one spectral acquisition per preparation and six repetitions of the data processing per spectrum.

Table 4. Histamine Content Found in Cheese Samples

cheese sample	histamine concentration (mg/kg)[a]	RSD (%)
Asiago	124.7 ± 0.7	2.2
Asiago DOP	186 ± 2	4.1
Emmental	323 ± 1	1.7
Pecorino Grotta	32.4 ± 0.3	3.3
Montasio DOP	594 ± 5	2.2
Parmigiano-Reggiano	32.1 ± 0.5	4.0
Tilsit	34.8 ± 0.4	4.4
Gorgonzola	115.6 ± 0.4	4.5
Pecorino Pienza	<LOD	
Bergkase	<LOD	

[a] Mean of 18 measurements per point, obtained from three different preparations per type of cheese, with one spectral acquisition per preparation and six repetitions of the data processing per spectrum.

was demonstrated with the spiked sample and is very satisfactory. Also, the precision obtained with cheese samples containing histamine is good.

The acquisition time depends upon the concentration of histamine: for levels higher than 100 mg/kg, a 16 min experiment is adequate; for levels of histamine ranging from 30 to 100 mg/kg, the experiment time is about 30 min, while for concentrations lower than 30 mg/kg (down to 4 mg/kg), 1 h and 20 min is necessary. A LOQ of 3.79 mg/kg can be obtained in acceptable times and is sufficiently low for most applications. This value can be further lowered by simply increasing the number of scans. Notably, this result is possible only using a pulse sequence that overcomes the dynamic range problems commonly encountered with sequences traditionally employed. The sequence that we employed allows, for the first time, an accurate NMR determination of histamine down to very low concentrations in a very short time. Such performance can be extended to the determination of other compounds in other matrices (*33*) and had not been achieved by NMR before.

To achieve an even lower LOQ, the sample preparation can be slightly modified by adding a lyophilization step of the extract. The subsequent dissolution in the minimum amount of water would allow for a LOQ of 1 mg/kg or lower to be reached.

An additional feature of this NMR method, which was not pursued in the present work, is the possibility to quantify also histidine, along with histamine. This development is in progress in our laboratory, as well as the comparison to official analytical methods. Other potential extensions of this NMR method can

be envisaged in the determination of histamine in other food matrices, either liquid or solid, or in biological samples, such as plasma and urine.

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