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QUANTITATIVE DETERMINATION OF N-ACETYLNEURAMINIC ACID BY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

An analytical method is described for preparing the trimethylsilyl derivative of N-acetylneuraminic acid (NANA) for quantitative gas–liquid chromatographic analysis. Satisfactory quantitation (RSD 1.6 %) on 200 μg amounts of NANA was obtained from a 2 % OV-17 column and flame ionization detector using an internal standard of *trans*-stilbene. The minimal detectable amount (MDA) was found to be about 8 ng of standard injected. The silylation of NANA was carried out in acetonitrile as solvent at 125° for 2 h using BSTFA as silylation reagent.

This procedure was successfully applied to the determination of NANA in K-casein (*ca.* 2 %). The NANA was hydrolyzed from the protein in 0.05 *N* HCl at 80° for 1.5 h and extracted with CH_3OH prior to silylation. These hydrolysis conditions were found to provide the maximum recovery of NANA from K-caseins. Any decomposition of NANA resulted in the appearance of an additional chromatographic peak in the 180° region of the chromatogram. The sialic acid values obtained by the proposed method were about 10 % higher than those of WARREN's colorimetric method. This is due in part to the hydrolysis conditions employed by the latter method. Two hundred microgram quantities of NANA were determined in K-casein by the proposed method with a relative standard deviation of 3.8 %.

An interesting by-product of this research was the appearance of a series of three compounds in the 200° region of the gas chromatograms of α_{s_1}- and β_s-caseins but not in K-casein. Identification of these compounds might benefit casein research with respect to the calcium sensitivity of the caseins (α_{s_1}- and β-) in which they are present.

This gas–liquid chromatographic method is offered as a more selective and probably more accurate alternative for determining sialic acids in biological samples and glycoproteins. It also demonstrates the capabilities of quantitative gas–liquid chromatographic analysis of higher molecular weight compounds.

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A more selective method was desired for the quantitative determination of sialic acids. The sensitivity (10 to 60 μg) of WARREN's assay[8] for sialic acids is excellent and only a few naturally-occurring compounds interfere. The chromogen in this procedure is β-formyl-pyruvic acid, which results from the periodate oxidation of compounds in the class of 2-keto 3-deoxy-sugars. However, the 2-deoxy-sugars (*e.g.*, 2-deoxyribose) yield malonaldehyde on periodate oxidation, which gives a color with 2-thiobarbituric acid. The absorption spectrum (max. 532 mμ) for the malonaldehyde chromophore overlaps that of β-formyl-pyruvic acid (max. 549 mμ). This necessitates a correction for 2-deoxyribose in WARREN's sialic acid procedure. In addition, since only the first four carbon atoms in the neuraminic acid molecule are involved in the color development, the procedure does not differentiate the various neuraminic acid derivatives (N-acetyl-, N-glycolyl- and N,O-diacetyl-).

The introduction of the trimethylsilyl group for making volatile TMS derivatives has made possible the analysis of higher molecular weight compounds by gas–liquid chromatography[2,4,5]. The TMS derivative has found very successful application in determining, by GLC, a large number of carbohydrate compounds[6], and BENTLEY *et al.*[1] reported obtaining a gas chromatographic peak for N-acetylneuraminic acid (NANA).

This paper reports the results of an investigation on the development of a quantitative assay method for NANA by gas–liquid chromatography using the TMS derivative. After accomplishing this with standard materials the method was applied to determining the NANA content of K-casein (*ca.* 2% NANA) and comparing this to colorimetric values. In addition, a further investigation was made into the effects of mild acid hydrolysis on NANA. The optimum working range of the method is 200–800 μg of NANA, however good results can be obtained at the 100 μg level.

EXPERIMENTAL

Instrumentation and reagents

An F and M Model 401 gas chromatograph and flame ionization detector was used. The gas chromatograph was equipped with a L and N Speedomax G recorder and Disc integrator, Model 203. The electrometer was a Barber Coleman, Model 5042-1.

Chromatographic conditions

Column temperature initial, 175°; final, 260°
Program rate 7.9°/min
Attenuation 1 K
Carrier flow, N_2 54 ml/min
Air (to detector) 350 ml/min
Hydrogen (to detector) 38 ml/min
Chart speed $^1/_3$ in./min.

A U-shaped borosilicate glass column packed with a substrate of 2.0 w/w % OV-17 on High Performance Chromosorb G was used for the chromatography. The OV-17 was obtained from Applied Science Labs, Inc., State College, Pa., and Chromosorb G was from Varian Aerograph, Walnut Creek, Calif. Nanograde acetonitrile

from Mallinkrodt Chemicals, St. Louis, Mo., was the silylating solvent. The silylating reagent, BSTFA ("Regisil" concentrate) was purchased from Regis Chemical Company, Chicago, Ill. Crystalline synthetic N-acetylneuraminic acid obtained from Sigma Chemicals, St. Louis, Mo., was used as the standard.

Procedures

A standard solution was prepared by dissolving 20 mg of NANA and 20 mg of *trans*-stilbene (I.S.) in 100 ml of reagent-grade CH_3OH giving a final concentration of 200 μg/ml of each. The desired amounts of standard solution were transferred into screw-topped culture tubes (Pyrex No. 9826, 16 × 75 mm) and taken to dryness on a roto-evaporator at 60°. During evaporation the tubes should be placed in a nearly vertical position to prevent coating of the material over a large interior surface area of the glass. One milliliter of acetonitrile and 0.3 ml of BSTFA were then added to the samples. The samples were mixed thoroughly and placed in a 125° oil bath for 2 h. They were shaken periodically for the first 5–10 min of heating. A good working concentration range is from 200–800 μg NANA/ml acetonitrile. It was necessary to dissolve the standards in CH_3OH rather than CH_3CN because of the low solubility of NANA in acetonitrile. After silylation the samples were allowed to come to room temperature, then injected (6 to 7 μl) in sufficient quantity to give a response of 500 to 600 counts.

To release quantitatively the NANA from the protein, 10 mg samples of K-casein were hydrolyzed in 2 ml of 0.05 *N* HCl for 1.5 h at 80°. After hydrolysis the samples were taken to visible dryness on a lyophilizer (approximate time: 7 h). NANA was extracted with 2 ml of warm (60°) methanol containing an approximately equal weight (NANA/I.S., 1:1) of *trans*-stilbene as internal standard. The extraction was conducted in the following manner. After adding the 2 ml of CH_3OH the samples were mixed for 5 to 10 min on a Vortex mixer, then centrifuged on a clinical centrifuge at 2,000 r.p.m. for 10 min and decanted into identical tubes avoiding the transfer of the insoluble protein. The extracts were then dried on a roto-evaporator in the same manner as described for the standards, then silylated in 1 ml of acetonitrile and 0.5 ml of BSTFA for 2 h at 125°. Excess BSTFA was used to react with the soluble protein that was carried over in the extraction. The concentration of NANA in the extract was about 200 μg/ml. It was necessary to extract NANA in this way to eliminate interference that would be caused during silylation by the fifty-fold excess of protein present.

Calculations

$$\text{(1) Relative weight response for NANA/I.S.} = \frac{\text{Area NANA}}{\text{Area I.S.}} \times \frac{\text{wt. I.S.}}{\text{wt. NANA}}$$

$$\text{(2) Grams of NANA} = \frac{\text{Area NANA} \times \text{wt. I.S.}}{\text{Area I.S.} \times 1.118}$$

RESULTS AND DISCUSSION

The *trans*-stilbene (retention temperature 210°) and NANA (retention temperature 255°) peaks are shown on the chromatogram in Fig. 1. With respect to

solubility, acetonitrile is a poor solvent for NANA but it was the only solvent in which a single chromatographic peak was obtained for NANA; dimethylformamide, dimethylacetamide and other more polar solvents resulted in two peaks. The relative weight response (RWR) for NANA/*trans*-stilbene was 1.118 ± 0.018 (ten independent analyses) calculated from disc integration data. The standard curve for NANA at concentrations between 200 and 800 μg/ml shows good linearity (Fig. 2). The RWR varied slightly due to instrumental changes; thus, it was necessary to run standards and calculate the RWR for each series of samples.

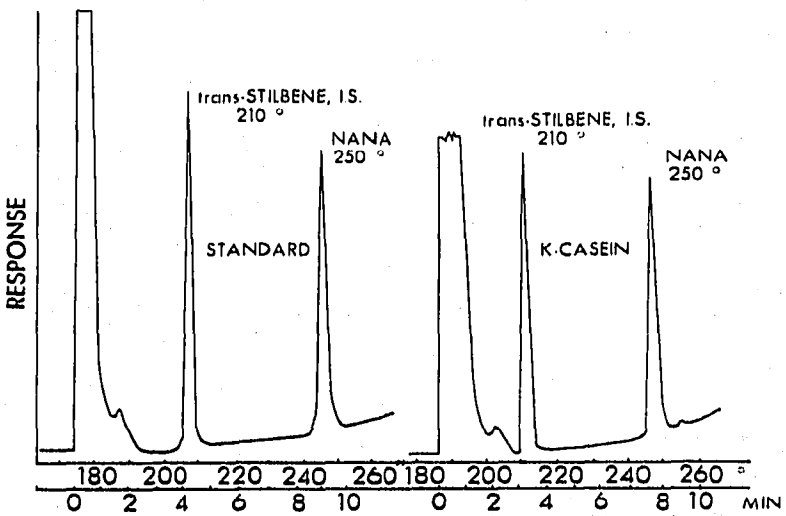

Fig. 1. GLC chromatograms of N-acetylneuraminic acid (NANA) and K-casein. Column: 2.0 w/w OV-17 on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D. glass; initial temperature: 175°; program rate: 7.9°/min; N_2 flow rate: 54 ml/min. Each peak represents 1.4 μg.

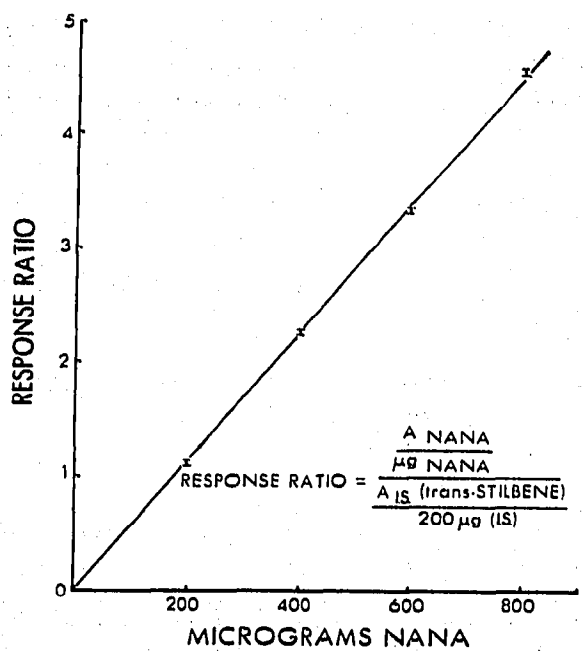

Fig. 2. N-Acetylneuraminic acid standard curve.

The effect of silylation temperature on RWR is shown in Fig. 3. Although considerably less silylation time is required at 150° it was not practical to use this high a temperature because of sample losses sustained from tube cap leakage. The silylated derivative showed good stability in closed tubes for periods up to one week. It was found that new rubber septa used for column injection produced peaks at chromatographic temperatures above 250° if they were not previously conditioned at high temperature for 24 h. Performance blanks containing only acetonitrile and BSTFA were analyzed periodically for extraneous chromatographic peaks and none were found.

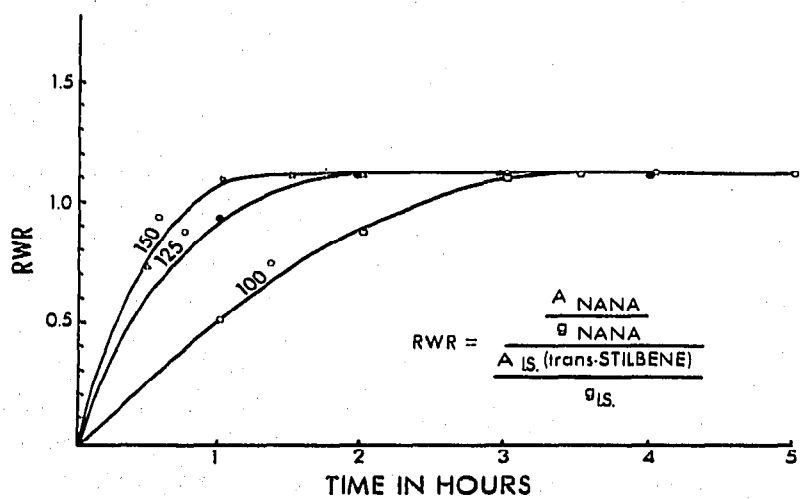

Fig. 3. Response as a function of silylation time and temperature.

The effects of acid hydrolysis on NANA were investigated and it was found that the RWR of the NANA standards decreased over 5 % after hydrolysis in 0.1 *N* HCl, 80°, for 1 h (Table I). The degradation of NANA was critically dependent on acid concentration. Decomposition of NANA was observed directly from the appearance of a new peak in the 180° region of the chromatograms. Lower concentrations of acid showed no effect on breakdown of NANA standards but low recoveries were obtained

TABLE I

RECOVERY OF N-ACETYLNEURAMINIC ACID AS A FUNCTION OF HYDROLYSIS TEMPERATURE, TIME, AND ACIDITY

	Temperature (°C)	*Time* (*h*)	*Acidity* (*N HCl*)	% *recovery*
NANA standard	60	1	0.2	86
	80	1	0.1	94
	80	1	0.75	95
	80	1.5	0.75	94
	80	1	0.05	101
	80	1.5	0.05	97
K-Casein[a]	80	1	0.05	87
	80	1.5	0.05	98
	80	2.0	0.05	96

[a] Based on NANA recovery from 0.05 *N* HCl, 80° and 1.5 h hydrolysis.

from the K-casein samples, indicating incomplete hydrolysis of NANA from the protein. From these experiments, the optimum hydrolysis conditions considering both parameters, loss due to decomposition of NANA and low values due to incomplete hydrolysis from the protein, were found to be 1.5 h, 80°, in 0.05 *N* HCl.

The GLC method was then applied for the analysis of sialic acid in an electrophoretically pure preparation of K-casein. The sample was found to contain 2.30 ± 0.09 w/w % NANA (Fig. 1, Table II). Table III gives the results obtained from the same K-casein by WARREN's method.

TABLE II

DETERMINATION OF SIALIC ACID IN K-CASEIN BY GAS–LIQUID CHROMATOGRAPHY

	Sample weight (mg)	*Area ratio NANA/I.S.*[a]	*Wt. NANA (μg)*	*% NANA*
NANA standard	0.200	1.000	200	100
	0.400	2.018	400	100
	0.600	2.964	600	100
K-Casein[b, c]	10	1.227	245	2.45
	10	1.194	239	2.39
	10	1.144	229	2.29
	10	1.096	219	2.19
	10	1.156	231	2.31
	10	1.100	220	2.20
	10	1.123	225	2.25
	10	1.193	239	2.39
	10	1.118	224	2.24
			Average	2.30 ± 0.09

[a] Corrected so that equal weights of NANA and I.S. give equal areas (*i.e.*, area I.S. × 1.118).
[b] 0.200 mg I.S. added to samples.
[c] All hydrolyzed in 0.05 *N* HCl, 1.5 h, 80°.

TABLE III

DETERMINATION OF SIALIC ACID IN K-CASEIN BY WARREN'S METHOD

	Sample (μg)	*549 mμ O.D.*	*NANA (μg)*	*% NANA*
NANA standard	10.8	0.460	10.8	100
	16.2	0.715	16.2	100
	21.6	0.940	21.6	100
K-Casein[a]	10[3]	0.860	19.7	1.97
	10[3]	0.870	19.9	1.99
K-Casein[b]	10[3]	0.900	20.7	2.07
	10[3]	0.880	20.2	2.02
K-Casein[c]	10[3]	0.910	20.9	2.09
	10[3]	0.910	20.9	2.09

[a] Hydrolyzed in 0.1 *N* H_2SO_4, 1 h, 80°.
[b] Hydrolyzed in 0.1 *N* HCl, 1 h, 80°.
[c] Hydrolyzed in 0.05 *N* HCl, 1.5 h, 80°.

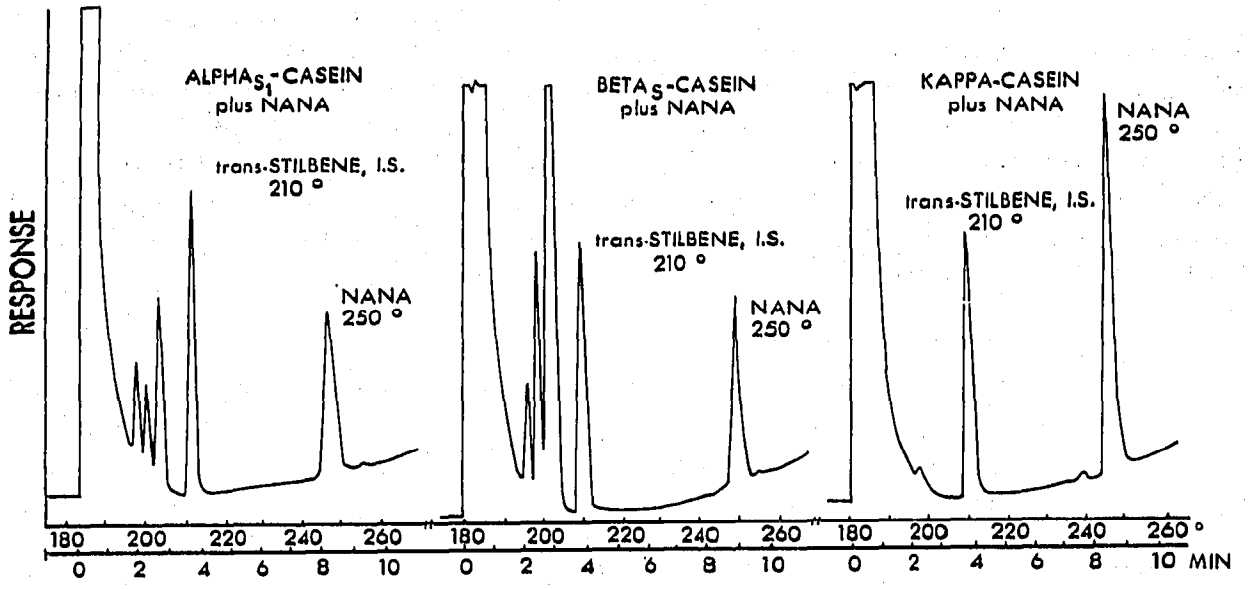

Fig. 4. GLC chromatograms of α_{S_1}-, β_S- and K-caseins with NANA added. Column: 2.0 w/w% OV-17 on 80/100 mesh H.P. Chromosorb G, 1.0 m × 4 mm I.D. glass; initial temperature: 175°; program rate: 7.9°/min; N_2 flow rate: 54 ml/min. Each peak represents *ca.* 1.4 μg.

In recovery experiments, samples of α_{S_1}- and β-caseins, containing no NANA of their own, were spiked with NANA and carried through the hydrolysis, extraction, and derivatization steps. In all cases, a recovery of better than 97% was achieved (Fig. 4). K-Casein was also spiked with NANA and a single chromatographic peak was observed, thus confirming the presence of the N-acetyl derivative of neuraminic acid in K-casein. Although only the N-acetyl substituted sialic acid was investigated in this research, other sialic acids could be analyzed in mixtures by this method.

Three major peaks (retention temperature 190° to 200°) were observed in the chromatograms of the α_{S_1}- and β_S-caseins but not in K-casein. β_S-Casein showed a large concentration of the component having the highest retention temperature. Although histidine, arginine, and cystine have retention temperatures in this region of the chromatogram, these peaks probably do not represent amino acids, otherwise, they would appear in K-casein as well. These are more than likely other types of carbohydrates known to be present in caseins. It is interesting to note that these compounds are present in the calcium-sensitive caseins, α_{S_1}- and β_S-, but not K-casein.

The minimal detectable amount (MDA), defined as the amount of substance needed to produce a signal to noise ratio of 2:1, was determined for the silylated NANA derivative and was found to be about 8 ng.

The OV-17 chromatographic column was found to be quite stable for several weeks of continuous use although the operating temperatures exceeded 260°. BSTFA was used as silylation reagent because its solubility in acetonitrile far exceeds that of ordinary BSA.

The colorimetric method of analysis gave lower values for NANA in K-casein than those obtained by gas–liquid chromatography. Obviously some of the NANA is decomposed by 0.1 *N* H_2SO_4 hydrolysis, even though NANA was reported to be stable under these conditions[7]. The colorimetric method probably is less sensitive to the partial decomposition of NANA because only a fragment of the molecule is re-

quired for color development; whereas, in the proposed method the entire molecule is derivatized. The GLC method has the advantage of selectivity, and is a convenient method for determining unbound and protein-bound sialic acids.

This procedure offers certain definite advantages over the colorimetric methods. Lower molecular weight carbohydrates do not interfere and the various neuraminic acid derivatives would be distinguishable from one another. This research also demonstrates the capability of gas–liquid chromatography as an analytical tool for higher molecular weight compounds. By controlling the extent of hydrolysis, a complete and rapid analysis of the amino acid and non-protein components; *e.g.*, carbohydrates, nucleic, acid constituents[3] and phosphate can be achieved on a single sample.

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