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# Quantitative Determination of Sialic Acids in Lipid Extracts: A Simple Procedure for Removal of Interfering Materials

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To determine sialic acid content in lipid extracts, a purification procedure is required. We describe here a chloroform–silica gel procedure that enables interfering materials to be removed and sialic acids to be quantitatively recovered from lipid extracts of human erythrocytes, rat brain, plasma, lung, heart and spleen. In this procedure, interfering materials were removed mainly in the silica gel after the colorimetric reaction, but also in the chloroform washing. Owing to its simplicity, this procedure may prove more advantageous than currently used methods.

Gangliosides are a group of glycosphingolipids which contain one or more sialic acid residues. The highest concentrations are generally found in the nervous tissue of higher vertebrates and they are also found in low amounts in many extraneural tissues and body fluids.[1–3] The content of the lipid-bound sialic acids is determined spectrophotometrically from either hydrolysates or tissue extracts, mostly by use of chemical reagents such as resorcinol–HCl,[4,5] thiobarbituric acid,[6,7] or diphenylamine.[8]

In nervous tissue[4,9] or platelets,[10,11] with relatively little interfering material, the resorcinol–HCl method may be used for sialic acid determination from lipid extracts. In other tissues, the presence of relatively large amounts of interfering materials limits its application to the lipid extracts. To remove these interfering materials, complicated combined procedures such as aqueous partition,[12,13] dialysis,[14] variable chromatographic procedures,[15–19] or others[20] have to be performed. Even so, correction for the interfering materials has frequently had to be made during dichromatic readings at 450–530 nm for the interfering materials and at 580 nm for sialic acids.[4,21,22] Furthermore, these procedures are time-consuming and suffer from a large variation in the ganglioside quantification due to incomplete removal of interfering materials, or to a loss of sialic acids by the multiple procedures.

In a project dealing with the influence of ganglioside content on cholesterol exchange between the vessel wall and blood cells, we found that it is possible to remove these interfering materials simply by adding chloroform and silica gel to the dried lipid extract of these tissues. The silica gel is removed and chloroform evaporated before the colorimetric reaction.

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*Materials.* Analytical reagent grade chemicals and solvents were used. All solvents and silica gel were obtained from Merck, D-6100 Darmstadt (Germany). All materials used as references were obtained from Sigma (St. Louis, USA). Methanol used for lipid extraction contained 0.05% BHT (butylated hydroxytoluene).

*Recommended procedure for removal of interfering materials and for sialic acid analysis from a lipid extract.* To test tubes containing dried lipid extract, were added 0.44 g silica gel H and 4 ml of chloroform. After mixing and centrifugation at 180*g* for 10 min, the chloroform was removed by evaporation under air until the silica gel was dry. To the silica gel were added 2.5 ml of water and 2.5 ml of resorcinol reagent.[4,5] The mixture was heated to 100 °C in a water bath for 15 min, then cooled in cold water for several minutes. After centrifugation, the chromagen-containing supernatant (3.4 ml) was transferred to another tube and the silica gel was washed consecutively with 5 ml then 2.5 ml of, water–resorcinol reagent (1:1 by vol). The pooled washings were mixed with 2 ml of butanol–butyl acetate (1:3 by vol) to partition the chromagen into the upper phase. The absorbance was measured 2 h later at 580 nm against a blank (with silica gel addition, washing, and heating) on a spectrophotometer (UV-260, Shimadzu).

A calibration curve was obtained using sialic acid standard (*N*-acetylneuraminic acids) with silica gel addition. Beer's law was valid up to 140 nM sialic acids. Day-to-day precision in triplicate analysis of total sialic acids in erythrocyte lipid extracts with the recommended procedure was 3.1% over 4 days.

0.44 g silica gel H was enough to remove the interfering materials from the lipid extract of 1 ml packed erythrocytes, 1 g rat lung, heart, spleen, or 2 g brain tissues.

Silica gel 60 H, silica gel 60 (230–400 mesh), and silica gel DG were all comparably effective in removing interfering material. For larger samples of most tissues, the addition of silica gel and water–resorcinol reagent in proportional quantities should be sufficient. For large amounts of erythrocyte extracts, excess pigments could be removed with a further prewash procedure using methanol–chloroform–water (2:1:1.4 by vol).[14] The pigment *per se* did not interfere with ganglioside analysis, but did affect the removal of interfering materials by competitive binding to the silica gel.

## Experimental and discussion

Aliquots of washed and packed erythrocytes, and ground samples of rat brain, lung, heart, liver and spleen, were extracted with 20 volumes of methanol–chloroform (2:1 by vol). Methanol was added 30 min before chloroform drop by drop while the tube was stirred on a mixer. Thirty minutes later, the extract supernatant was transferred to another tube and centrifuged at 1800*g* for 20 min. The supernatant was evaporated on a rotary evaporator, redissolved in 2 ml of methanol–chloroform (2:1), transferred to another tube and evaporated under air.

A portion of rat brain extracts was purified with a combined procedure of column chromatography[23] and partition and dialysis[14] (column chromatography–partition–dialysis).

Freshly extracted samples were usually used for ganglioside analysis. If this was not possible, lipid extracts were stored at −20°C until analysed. Values were expressed as mean ± SD.

*Comment on the procedure.* To test the efficiency of the recommended procedure in the removal of interfering material and in the recovery of gangliosides, the sialic acid content was also determined in standard solution (totally 60 nM sialic acid), and in aliquots of dried crude erythrocyte lipid extracts, as well as in the extracts after purification with either the recommended procedure or with the combined column chromatography–partition–dialysis procedure. Chromagen absorbance in the upper phase was scanned at wavelengths of 400 to 700 nm. In the recommended procedure, the chromagen was also determined from the chloroform washing and from the silica gel (see Fig. 1).

*(1). Removal of interfering materials.* It is not possible to determine the sialic acid content directly from an erythrocyte lipid extract without the removal of interfering materials (Fig. 2a, compared with sialic acid standard spectra of 2c). With the recommended procedure, interfering materials were substantially removed into the silica gel (Fig. 2b) after the chromagen-containing supernatant had been transferred to another tube, as evidenced by the reduced peaks of the final chromagen at 400 nm to 500 nm (Fig. 2e). Part of the interfering materials was also removed in the chloroform washing (Fig. 2f). A small portion of the interfering material may also remain in the chromagen-containing solution after partition. The nature of the interfering materials in the erythrocytes is not clear. The materials that have peaks around 400 to 500 nm might represent hexoses, pentoses, glucuronic acid and 2-deoxyhexoses.[4]

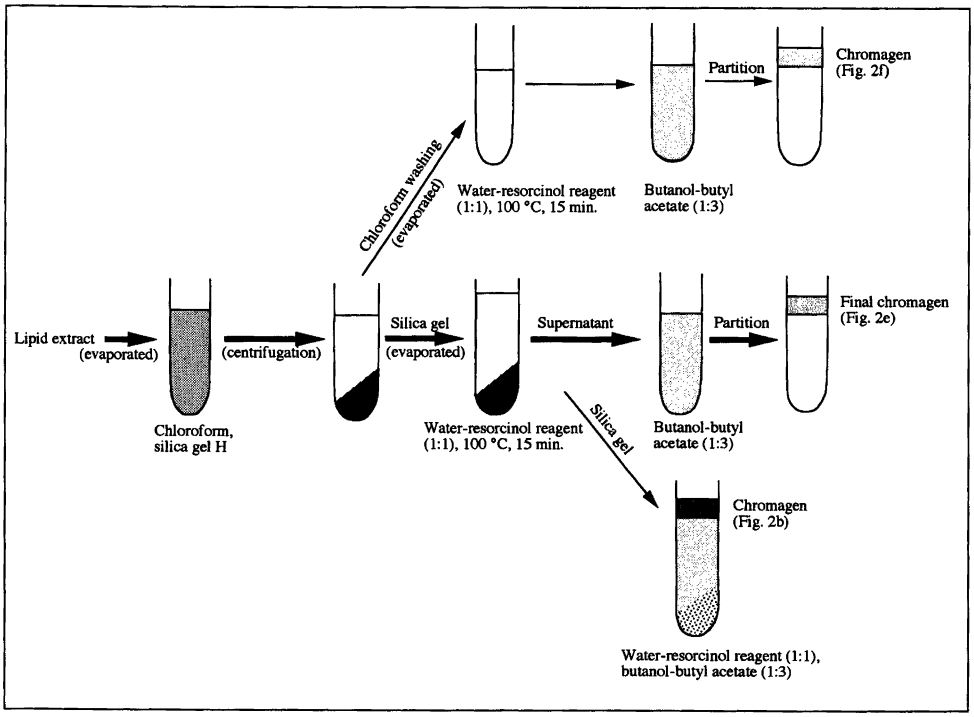


*Fig. 1.* Recovery of chromagen from different steps of the recommended procedure.

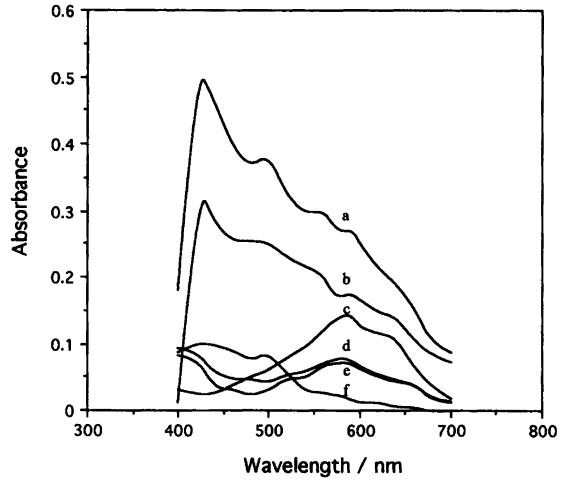


*Fig. 2.* Spectra of chromagen absorbance in sialic acid analysis of crude, purified erythrocyte lipid extracts and of sialic acid standard. Erythrocyte lipid extract was centrifuged. The supernatant was evaporated on a rotary evaporator, redissolved in methanol–chloroform (2:1), transferred to another tube and evaporated again. The chromagen was obtained with the procedures described in Fig. 1. a, Total chromagen from crude erythrocyte extract without purification. b, Chromagen recovered in silica gel. c, Chromagen of sialic acid standard (60 nM in water). d, Final chromagen purified by the column chromatography–partition–dialysis method. e, Final chromagen purified by the recommended method: 0.44 g silica gel and 4 ml of chloroform were added to tubes. After centrifugation and removal of chloroform, the silica gel was made solvent-free by evaporation and mixed with water–resorcinol reagent (1:1). The mixture was heated to 100°C for 15 min. After centrifugation, the supernatant was transferred to another tube and mixed with 2 ml of butanol–butyl acetate (1:3). The absorbance was measured 2 h later at 580 nm against a similarly prepared blank but no extract addition. f, Chromagen lost in chloroform washing. Spectra were scanned in triplicate and the mean value taken. Absorbance values were corrected for the same volume of water–resorcinol reagent.

*(2). Recovery of gangliosides from silica gel after chloroform washing.* No sialic acids (free and lipid-bound sialic acids) were lost in the chloroform washing, since the solubility of sialic acids or lipid-bound sialic acids is poor in non-polar solvents.[24] Even if the lipid-bound sialic acids were in a soluble form in the chloroform, they would be completely recovered in the subsequent silica gel treatment (Table 1). Moreover, using a sialic acid standard (106 nM), or a purified rat brain lipid extract (80 nM lipid-bound sialic acids), no sialic acids were apparent in the washings, since no purple–blue chromagen was seen on the sialic acid analysis on the dried washing, and no purple–blue band was visualised on the thin-layer chromatographic plate with a sensitivity of 2 nM sialic acids (Rosengren method[25]). The chromagen present in the chloroform washings, appearing at 550 nm to 600 nm (Fig. 2f), was therefore not interpreted as representing sialic acids.

The recovery of sialic acids in a single pool of erythrocyte extracts with the recommended procedure was $18.5 \pm 0.7$ nM ml$^{-1}$ packed cells ($n = 5$), and that with the available column chromatography–partition–dialysis procedure was $20.1 \pm 1.6$ nM ml$^{-1}$ packed cells ($n = 5$). Although the difference between the two values did not become statistically significant (Student unpaired $t$-test), the chromagen spectra suggest that the recommended method (Fig. 2e) yielded better purification than the old method (Fig. 2d).

Of the solvents tested (Table 1), butyl acetate also yielded 100% recovery of gangliosides in the silica gel, but purification was less good than with chloroform. The data in Table 1 indicate that the binding of gangliosides to silica gel may not simply be related to the polarity of the solvent, as silica gel binds a similar amount of ganglioside (72% and 69%, respectively) in toluene (polarity 0.099) and ethanol (polarity 0.654).

*(3). Recovery of chromagen from final silica gel (Fig. 2b).* After heating and transfer of the chromagen-containing supernatant, the remaining solution in the silica gel contained some chromagen (Fig. 2b), which could be recovered either by applying more water–reagent solution be-

*Table 1.* Recovery of sialic acid in silica gel (expressed as a percentage of control values, $n=3$, mean$\pm$SD).[a]

| Solvents | Recovery | Solvents | Recovery |
|---|---|---|---|
| Chloroform | 100.2(29) | Toluene | 72.1(14) |
| Butyl acetate | 99.6(19) | Ethanol | 69.2(9) |
| Isopropyl alcohol | 96.4(19) | Cyclohexane | 67.1(4) |
| Isopropyl alcohol–chloroform 1:1 | 90.1(21) | Methanol–chloroform 1:1 | 57.5(6) |
| Isopropyl alcohol–cyclohexane 1:1 | 85.6(21) | Xylene | 49.8(3) |
| Isopropyl alcohol–hexane 2:3 | 84.3(13) | Acetone | 45.3(8) |
| Butanol | 84.1(12) | 70% ethanol | 44.2(3) |
| Methanol–chloroform 1:2 | 80.3(9) | Methanol–chloroform 2:1 | 28.3(9) |
| Hexane | 75.8(9) | Methanol | 14.5(4) |

[a] Aliquots purified rat brain lipid extract (50 µl containing 50 nM sialic acids in methanol–chloroform 2:1) were mixed with 4 ml of different solvents. Silica gel H (0.22 g) was then added. After centrifugation and transfer of chloroform to another tube, silica gel was evaporated and sialic acid content was determined (see the text). Results were compared experimentally with the mean sialic acid content in the control, without silica gel addition. Values in parentheses are standard deviations.

fore the colorimetric reaction, or by washing the silica gel with water–resorcinol reagent (1:1). The amount of sialic acid chromagen lost in the silica gel was proportional to the solution volume left in the gel. The initial colorimetric reaction conducted in 5 ml of water–resorcinol reagent recovered 69.2 ± 2.1% ($n = 4$) of total chromagen of sialic acid standard in the upper phase (chromagen absorbance multiplied by the total volume of the upper phase). When the silica gel was washed first with 5 ml, then with 2.5 ml of the water–resorcinol reagent (1:1), total chromagen recovery reached 97.1 ± 0.5% ($n = 4$). Washing with only water is not recommended, since this resulted in a 10% to 20% lower recovery of the chromagen. It is possible that the addition of more water changed the pH of the chromagen-containing solution and shifted the binding of sialic acid chromagen to the silica gel.

*(4). Partition of the chromagen into upper phase.* For this, Miettinen[5] used butanol–butyl acetate (15:85 by vol). Using more butanol (butanol–butyl acetate 1:3 by vol), we obtained a 20–30% greater absorbance of the sialic acid standard. This may have been due to a shift of the chromagen from red–purple to blue (wave peak at 580 nm) on further butanol addition. Furthermore, butanol–butyl acetate (1:3) extracted 20 to 40% less interfering material-derived chromagen than did butanol–butyl acetate (15:85) (data not shown). Both with butanol–butyl acetate (1:3) and with butanol–butyl acetate (15:85), the absorbance of sialic acid standard decreased by an average of 9.2% ($n = 3$) within the first hour, 5.7% within the second hour, then by 1.9% per hour for the next 8 h.

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