Journal of Chromatography, 129 (1976) 31–39

CHROM. 9232

LIQUID CHROMATOGRAPHY OF NEOHESPERIDIN DIHYDROCHALCONE

ROLF SCHWARZENBACH
Givaudan Research Company Ltd., Ueberlandstr. 138, CH-8600 Dübendorf (Switzerland)
(Received March 22nd, 1976)

SUMMARY

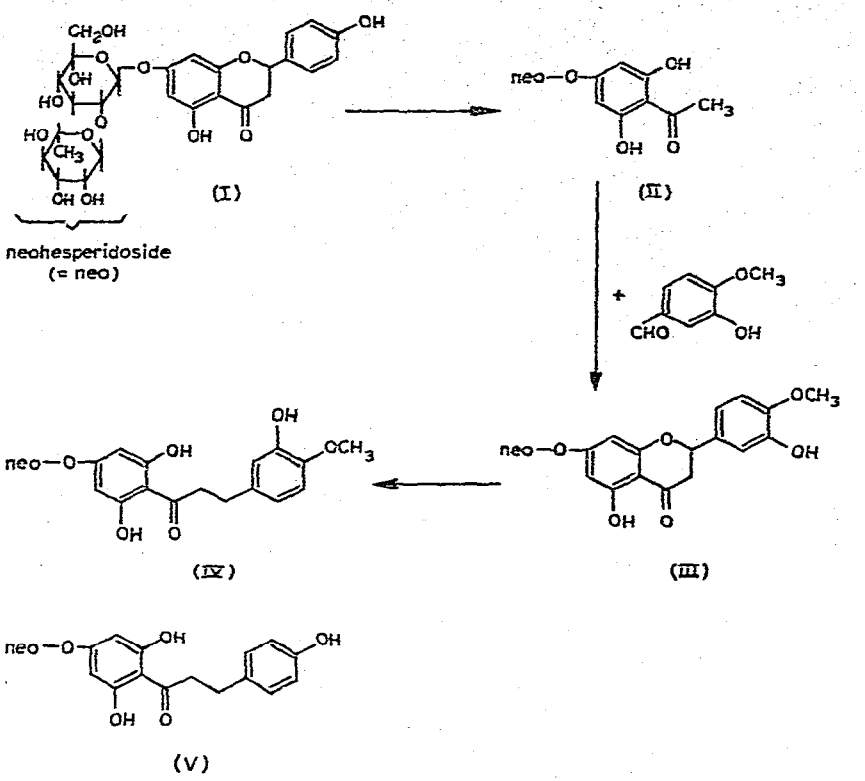
The determination of a number of impurities in neohesperidin dihydrochalcone is described, as well as the determination of neohesperidin dihydrochalcone in food products. A reversed-phase chromatographic system is used, with octadecyltrichlorosilane-treated silica gel as chemically bonded stationary phase and methanol–water as mobile phase; a detailed procedure for preparing the packing material is given, and the dependence of the amount of bonded stationary phase on the humidity of the silica gel before the bonding reaction is shown. The glass column was packed at 350 atm in a steel pressure vessel, with use of a slurry-packing technique; details are given of the pressure vessel and of the packing method. Chromatograms are reproduced to show the performance of such columns in this type of analysis.

INTRODUCTION

Neohesperidin dihydrochalcone (NHDC) (IV) is a non-nutritive, intense sweetener of potential interest to the food industry. It is approximately 2000 times sweeter than sugar and might be used in soft drinks, yoghurt, chewing gum and many other food products in which the use of sugar is undesirable. NHDC is prepared by treating naringin (I), the bitter principle of grapefruit, with alkali to form phloroacetophenone-4-β-neohesperidoside (II), which undergoes aldol condensation with isovanillin to form neohesperidin (III). The dihydrochalcone derivative NHDC (IV) is obtained by hydrogenation of neohesperidin[1,2].

As well as NHDC, the final product may contain small amounts of I, II, III and naringin dihydrochalcone (V), depending on the reaction conditions and on the extent of purification.

These closely related compounds have been separated by thin-layer chromatography (TLC) on polyamide, by paper chromatography and by paper electrophoresis[3]. Plastic foils pre-coated with cellulose were used by Robertson *et al.*[2] to separate all but NHDC and V. In the absence of interfering compounds, Linke[4] determined NHDC quantitatively by measuring (at 405 nm) the absorption of its complex with 2-aminoethyldiphenylborate. As TLC is rather cumbersome and insufficiently accurate for the quantitative determination of NHDC and related products, we have developed



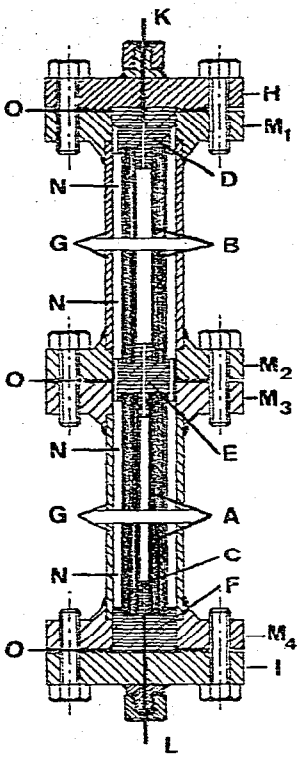
a liquid chromatographic (LC) method for the determination of impurities in NHDC and of NHDC in food products.

EXPERIMENTAL

Apparatus

The LC apparatus was constructed from commercially available components and has been described previously[5]. The UV detector used to monitor the eluent at 280 nm was an LDC Model 1285 (Laboratory Data Control, Riviera Beach, Fla., U.S.A.).

The pressure vessel used to pack the glass column is made from stainless-steel tubing and shown in Fig. 1. For practical purposes, this vessel is assembled in two sections. The end flanges (M), each with four symmetrically positioned holes, are welded to the ends of the stainless-steel tubes. The chromatographic column (A) and the reservoir tube (B) are high-precision KPG-tubes® with an O.D. of 12 mm and an I.D. of 3 ± 0.02 mm and 5 ± 0.02 mm, respectively; these glass tubes are available in standard lengths of 250 mm and 500 mm (Gebr. Möller, Zürich, Switzerland). The inlet plug (D), the outlet plug (F) and the union (E) are made of PTFE. The pressures in the space N and column A are equalized by means of a channel intersecting at right angles with the axial bore of the inlet plug. The end of column A is closed by a PTFE frit (C). A diaphragm-type pulsating pump equipped with a 3-mm plunger (Orlita Dosiertechnik, Giessen, G.F.R.) is used to force the slurry into the chroma-



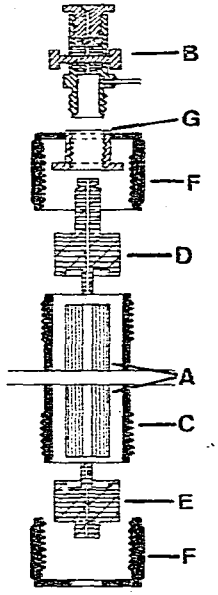


Fig. 1. Cross-section of apparatus. A = Chromatographic column; B = reservoir tube; C = PTFE frit; D = inlet plug; E = union; F = outlet plug; G = pressure vessel; H = head flange; I = bottom flange; K = inlet capillary; L = outlet capillary; M_1–M_4 = end flanges; N = space; O = PTFE gasket.

Fig. 2. A = Chromatographic column; B = LC-inlet; C = aluminium jacket; D = PTFE inlet plug; E = PTFE outlet plug; F = nut; G = steel interface.

tographic column; the maximum flow-rate of this pump is 630 ml/h at a maximum output pressure of 350 atm. The pressure in the system is monitored by a bourdon-type pressure gauge (Type 232, NG 100, Manometer, Lucerne, Switzerland). All connecting tubes and fittings (Swagelok) are of stainless steel.

Materials

LiChrosorb SI 60, an irregular silica with a particle diameter of 5 μm and an average pore diameter of 60 Å (E. Merck, Darmstadt, G.F.R.) was chosen as a basis for preparing the C_{18} packing material. Toluene (Merck), acetone (Merck) and methanol (Fluka, Buchs, Switzerland) were of analytical-reagent grade and were used without further purification. The octadecyltrichlorosilane was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was used as received. The lithium chloride used to prepare a saturated solution was of purum quality (Fluka).

Preparation of column-packing material

The chemical bonding of octadecyl groups to silica gel has been described by

several authors[6–9], and as shown by Majors and Hopper[6], the humidity of the gel influences the type of bonding. We prepared our packing material in the following manner. Before the bonding reaction, the silica gel is conditioned to a relative humidity of 15% by placing it over a saturated solution of lithium chloride in a desiccator. A 5-g portion of this silica was placed in a 250-ml round-bottomed flask, and 100 ml of a 5% solution of octadecyltrichlorosilane in toluene were added. The resulting slurry was de-gassed for 15 min at room temperature by carefully evacuating the reaction vessel, then the vacuum · placed by nitrogen, and the flask was immersed in a water bath at 90°. The flas' owly rotated to prevent sedimentation and kept at this temperature for 5 h, the ..e .action mixture was cooled to room temperature and the slurry was filtered on a glass frit. The product was washed at least five times with toluene and then rinsed with acetone, and the chloro-groups of the stationary phase were converted into methoxy-groups by washing the material several times with methanol until no hydrochloric acid could be detected. It was then rinsed with acetone and dried, at room temperature, by passage of dry nitrogen through it.

Preparation of the column

The high-pressure slurry-packing technique has been used successfully to produce efficient columns with small particles for high-performance LC[10–12]. Since glass columns cannot withstand the filling pressures (over 350 atm), packing is carried out in a pressurised vessel (Fig. 1) in which the pressure outside the column is equal to the filling pressure. The column A and the reservoir tube B are connected by the union E, frit C is inserted, and outlet plug F is attached. The two halves of the pressure vessel are connected with four bolts, and an annular gasket (O) made from 1-mm PTFE sheet, is used for sealing. The column A, with the connected reservoir B, is pushed into the pressure vessel (G), the bottom flange (I) is bolted to the flange M_4, and outlet capillary L is inserted through flange I into plug F. The connection of capillary L in plug F must be very tight in order to prevent liquid from leaving the pressure vessel without passing through column A.

The column, reservoir and space N between them and the vessel wall are now filled with methanol. The methanol in the reservoir is then taken out and added to approximately 3 g of the packing material in a beaker to form a slurry, and this is poured into the reservoir tube, care being taken to minimize the formation of bubbles. Inlet plug D is then pushed into the reservoir tube, and flange H is bolted to flange M_1, the joint being sealed with a PTFE gasket. Finally, the pump is connected with inlet capillary K, through which the whole system is pressurized with methanol as rapidly as possible. The slurry is thereby forced from the reservoir into the column; usually, 2 h of pumping at 350 atm are sufficient to pack the column.

Instead of methanol, any other solvent of medium or high density may be used with practically no adverse effect on the plate number, provided that the time between filling the reservoir and pressurizing the whole system is short.

The connection of the glass column to the LC inlet (Precision Sampling, Baton Rouge, La., U.S.A.) is made by means of a PTFE plug, as shown in Fig. 2; the purpose of the aluminium jacket is to squeeze the PTFE parts and the glass column tightly together. The inlet capillary of the LC detector is pushed directly into the outlet plug.

Sample preparation for determination of NHDC

Soft drinks. The analyses of a tonic and a cola product are quoted as illustra-

TABLE I

COMPOSITIONS OF SOFT-DRINK SAMPLES

Component	*Content in tonic, %*	*Content in cola, %*
Sugar syrup (85° Brix)	6.3	3.4
Fructose syrup (70° Brix)	—	3.0
Citric acid	0.9	—
Phosphoric acid	—	0.09
Quinine hydrochloride	0.6	—
Potassium sorbate	0.01	0.01
Sugar colour ("Maizena")	—	0.24
Caffeine	—	0.02
Flavouring	0.06	0.05
D-Gluconolactone	0.02	0.02
NHDC	0.0016	0.0018
Ethanol	0.1	0.1
Water	92.0	93.1

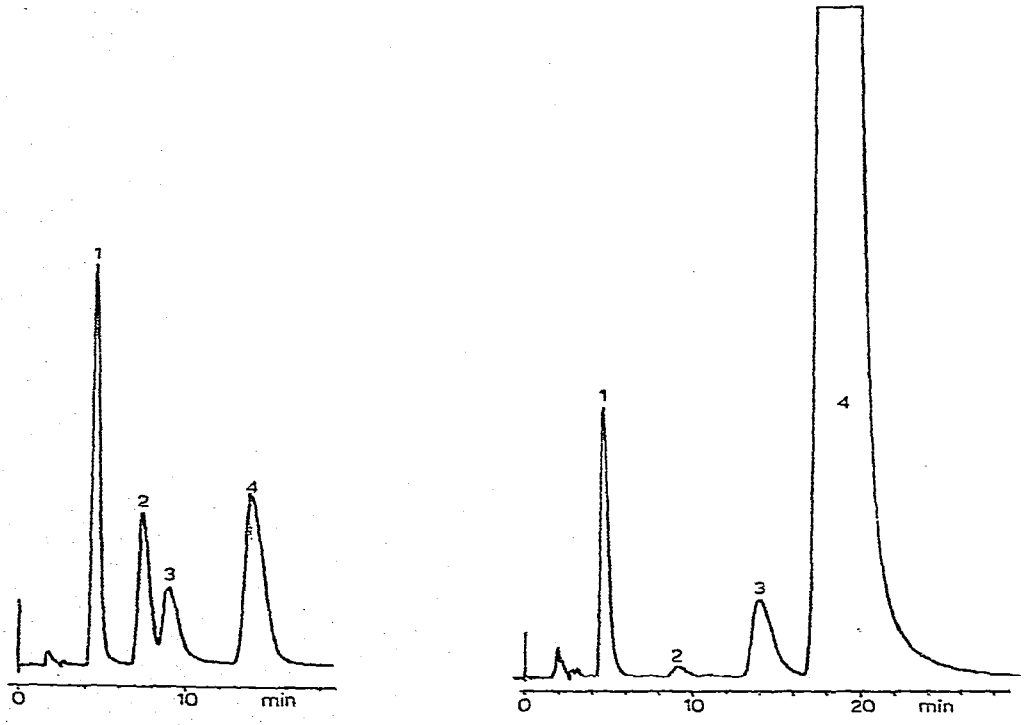


Fig. 3. Separation using 40% methanol–water as mobile phase at 0.8 ml/min. Peaks: 1 = phloroacetophenone-4′β-neohesperidoside (400 ng); 2 = naringin (300 ng); 3 = neohesperidin (200 ng); 4 = naringin dihydrochalcone (500 ng).

Fig. 4. Separation using 40% methanol–water as mobile phase at 0.8 ml/min. Peaks: 1 = phloroacetophenone-4′β-neohesperidoside (0.2%); 2 = neohesperidin (0.02%); 3 = naringin dihydrochalcone (0.25%); 4 = NHDC (99%).

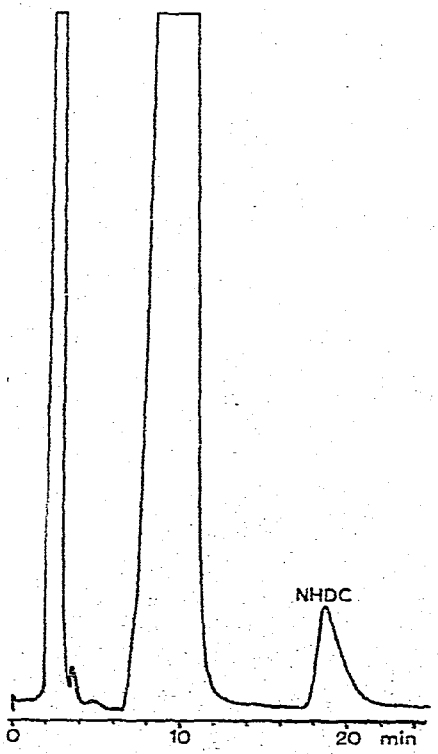
TABLE II

QUANTITATIVE DETERMINATION OF NHDC IN SOFT DRINKS

Soft drink	*NHDC added, μg/ml*	*NHDC found, μg/ml*
Tonic		
No. 1	6.3	6.3
No. 2	10.4	10.5
No. 3	16.7	16.3
Cola		
No. 1	11.5	12.8
No. 2	15.6	16.0
No. 3	7.3	8.2

tions of the method. The soft drinks analysed had the compositions shown in Table I. The only preparation of the sample consisted in shaking it well, in order to remove all carbon dioxide. Samples (each 20 μl) were analysed directly, a sample of pure NHDC being used as external standard under identical conditions.

Yoghurt. A strawberry-flavoured skimmed-milk yoghurt containing 7% of fresh strawberry pulp and 75 ppm (75 μg/g) of NHDC was analyzed. To 5 g of this yoghurt in a centrifuge tube were added 5 ml of acetone (puriss p.a.), the contents



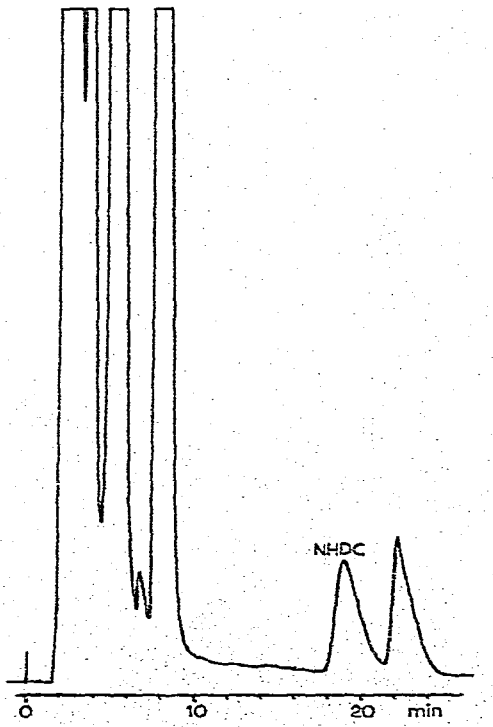


Fig. 5. Chromatogram of 20 μl of tonic; 40% methanol–water as mobile phase at 0.8 ml/min.

Fig. 6. Chromatogram of 20 μl of cola; 40% methanol–water as mobile phase at 0.8 ml/min.

were mixed vigorously for 3 min, and the tube was then centrifuged at 4500 *g* for 10 min. The clear acetone layer was transferred to a 10-ml volumetric flask, the residual material was similarly extracted with another 5 ml of acetone, and the combined acetone layers were diluted to 10 ml with acetone. A 10-μl portion of this solution was injected into the liquid chromatograph; the NHDC content was determined by using pure NHDC as external standard under identical conditions.

Chewing gum. A 1-g portion of chewing gum was cut in small pieces and dissolved in 10 ml of chloroform (Siegfried, Zofingen, Switzerland) by stirring with a magnetic stirrer at room temperature for 40 min. The turbid solution was then extracted with three 5-ml portions of water, the combined aqueous extracts were centrifuged for 5 min at 4500 *g*, and 10 μl of the clear supernatant layer were injected into the liquid chromatograph. Pure NHDC analyzed under the same conditions was used as external standard. The chewing gum used to evaluate this procedure had the following percentage composition: Yalaco chewing-gum base, 30.0; sorbitol, 64.5; mannitol, 5.0; anise flavour, 0.5; and NHDC, 0.03.

RESULTS AND DISCUSSION

The separation of a standard mixture of the common impurities of NHDC is shown in Fig. 3. By reducing the methanol content of the mobile phase, the separation

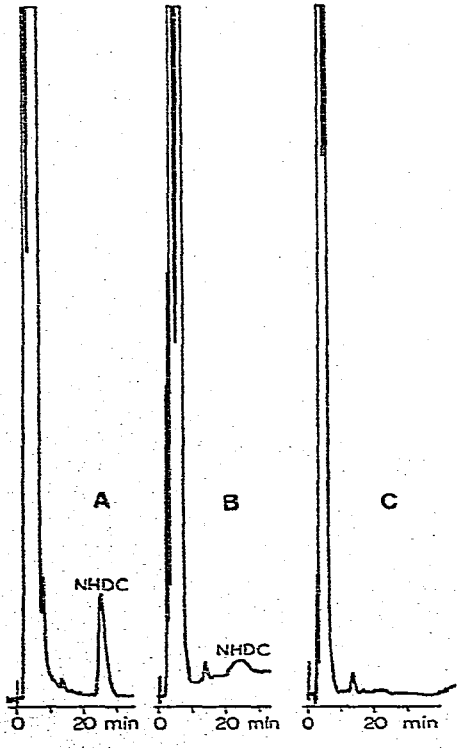


Fig. 7. Chromatograms of yoghurt extracts: A = first extraction; B = second extraction; C = third extraction. Separation using 40% methanol–water as mobile phase at 0.6 ml/min.

of naringin from neohesperidin can easily be improved, but this is not usually necessary, because, after hydrogenation, naringin is completely converted into naringin dihydrochalcone. A typical chromatogram of NHDC (purity 99%) is shown in Fig. 4. For the determination, an external-standard method is used, peak heights in the sample chromatogram being compared with those in chromatograms of reference solutions analysed under identical conditions.

The results of determinations of NHDC in three samples of tonics and three of cola are shown in Table II, and Figs. 5 and 6 represent typical chromatograms of such soft-drink analyses. With samples of tonic, the analysis time could be reduced by adding more methanol to the mobile phase or by using chemically bonded phenyl instead of octadecyl groups as stationary phase.

Four independent analyses of a yoghurt sample containing 75 μg/g of NHDC gave results of 74, 80, 72 and 73 μg/g of NHDC, an average of 74.7 μg/g. The chromatograms of the extracts after one, two and three extractions with acetone (see Fig. 7) show that two extractions are sufficient to remove the NHDC. In our experiments, the results of single determinations differed by less than 10% from the theoretical

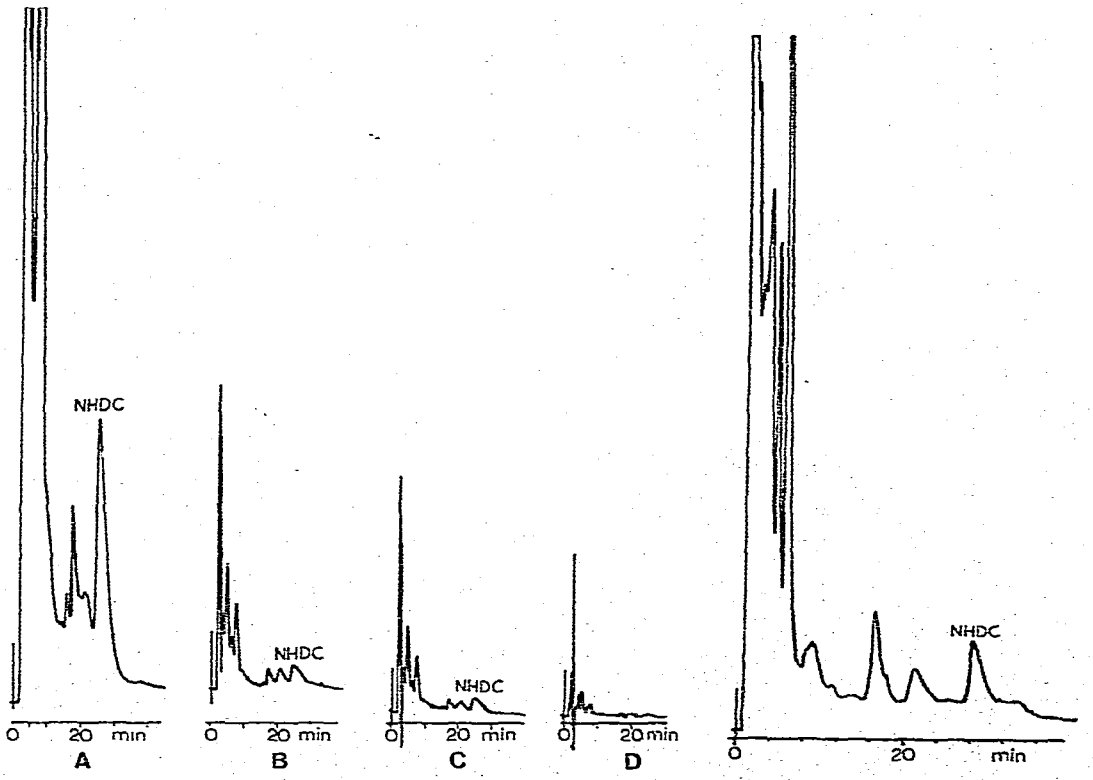


Fig. 8. Chromatograms of chewing-gum extracts. A = first extraction; B = second extraction; C = third extraction; D = fourth extraction. Separation using 40% methanol–water as mobile phase at 0.6 ml/min.

Fig. 9. Chromatogram of 10 μl of combined chewing-gum extracts. Separation using 40% methanol–water as mobile phase at 0.6 ml/min.

value, and 1 μg/g of NHDC could be clearly distinguished from the background fluctuations.

Within the range 0 to 2000 μg of NHDC per gram of chewing gum the proposed extraction method gave peak heights proportional to the NHDC concentration. As shown in Fig. 8, three extractions are necessary for quantitative determination. The yield of the first extraction could be improved by using aqueous 0.1 *N* sodium hydroxide instead of pure water; however, in some instances, this led to extraction of an additional impurity, which interfered with the determination. The chromatogram of a typical determination is shown in Fig. 9. The results of single determinations were within $\pm 10\%$ of the theoretical values.

The same chromatographic system can be used for all these separations, with 40% methanol in water as mobile phase. The preparation of the packing material is simple and reproducible. It is essential to moisten the silica gel before the bonding reaction; the use of dry silica gel was the least satisfactory in every aspect. By conditioning the gel to a definite humidity, the amount of bonded stationary phase can be increased. This, in turn, increases the capacity of the column, which is desirable for the relatively large amount of sample (*e.g.*, soft drink) injected. The humidity controls the extent of the polymerization, since the silane contains three reactive sites, and cross-linking and/or linear polymerization reactions may occur. From experiments, we know that some surface hydroxy-groups still remain accessible, but we found it unnecessary to mask them by treating the packing material with chlorotrimethylsilane.

ACKNOWLEDGEMENTS

I thank Miss K. Schneider for technical assistance and Mrs. A. Ehrat for preparing some of the samples.

REFERENCES

1 R. M. Horowitz and B. Gentili, *U.S. Pat.*, 3,087,821 (1963).
2 G. H. Robertson, J. P. Clark and R. Lundin, *Ind. Eng. Chem., Prod. Res. Develop.*, 13 (1974) 125.
3 B. Gentili and R. M. Horowitz, *J. Chromatogr.*, 63 (1971) 467.
4 H. A. B. Linke, *Hauptversammlung 1975, Ges. Deutscher Chemiker, Köln*, September 1975.
5 R. Schwarzenbach, *J. Chromatogr.*, 117 (1976) 206.
6 R. E. Majors and M. J. Hopper, *J. Chromatogr. Sci.*, 12 (1974) 767.
7 A. Pryde, *J. Chromatogr. Sci.*, 12 (1974) 486.
8 J. J. Kirkland, *Chromatographia*, 8 (1975) 661.
9 D. G. I. Kingston and B. B. Gerhart, *J. Chromatogr.*, 116 (1976) 182.
10 J. J. Kirkland, *J. Chromatogr. Sci.*, 10 (1972) 593.
11 R. E. Majors, *Anal. Chem.*, 45 (1973) 755.
12 W. Strubert, *Chromatographia*, 6 (1973) 50.