THE RELATIONSHIP BETWEEN SOLID SUPPORT, COLUMN EFFICIENCY, AND STEROL QUANTITATION BY GAS CHROMATOGRAPHY

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Since the first report of gas chromatographic separation of C-27 sterols there have been several papers describing quantitative methods. Although most authors reporting sterol quantitation have used argon ionization detectors, results and interpretations have varied. SWEELEY AND CHANG[1] first pointed out a need for individual calibration of each sterol in a mixture. Within limits these authors and others[2] found C-27 sterol responses to be linear in an ionization detector. Recently ROSENFELD *et al.*[3] have shown non-linear responses with cholesterol and coprostanol. My own experience has been that while these substances are never truly linear, under certain conditions they approach linearity[4] and because of relationships between response, retention time and oxygenation, quantitation of a variety of sterols may be accomplished using appropriate standards. I have recently observed that when the column efficiency is changed, molar responses and limits of linearity for sterols may be markedly altered. This can be advantageous, but calls for the modified approach to quantitation of sterol mixtures reported below.

METHODS

Apparatus and conditions are similar to those previously used[4]. Briefly they are Barber-Colman Model 10 argon capillary ionization detector, 6 ft., 1/4 in. I.D. column. Column temperature 250°, flash heater 270°, detector 260°, anode voltage 1500. Two column packings were used, Chromosorb-W (120–140 mesh)** and Gas Chrom-P (80–100 mesh)***. The former packing gives a lower efficiency column and the latter packing gives a higher efficiency one. Both packings were presiliconized and coated with 1% SE-30 silicone gum rubber†[5]. Theoretical plates were calculated by the method described by HARDY AND POLLARD[6]. Areas were calculated as previously described[4]. A standard mixture of sterols was used throughout the study except where noted. This contained in each milliliter of methylene chloride, cholestane 0.2 mg, cholestan-3β-ol 0.6 mg, cholestane-3β,7α-diol 1.0 mg, and cholestane-3β,5α,6α-triol 5.0 mg. Cholestan-3β-ol serves as a satisfactory substitute for cholesterol and

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appears to be more stable in solution. Methylene chloride is preferred as a solvent because there is minimal tailing of solvent peaks.

RESULTS AND DISCUSSION

Earlier work[4] was done using Chromosorb-W (120–140 mesh) with a 1% coating of SE-30. When the column support was changed to Gas Chrom-P (80–100 mesh) a large increase in efficiency was obtained so that theoretical plates were about twice those of previous columns. The increased column efficiency markedly changed compound thresholds and linear ranges for those compounds with oxygen functions. The differences became larger as the number of oxygen molecules incorporated into these steroids increased. More highly oxygenated compounds such as cholestanediols and cholestanetriols quantitated well with smaller amounts of material than were previously used. This was very helpful in the study of fecal sterol mixtures in which these compounds were present in relatively small amounts. Paradoxically, the quantitation of simple compounds such as cholesterol, became more complicated since useful linearity decreased as column efficiency increased. When increased column efficiency emphasized the non-linearity of the argon detector to the point that a good linear range for comparison with an internal standard such as cholestane was difficult to determine, changes in my original method of quantitation of complex mixtures of fecal sterols[4] were essential since that method was based on the broad linear ranges afforded by low efficiency columns. Furthermore, as will be shown below, responses and linear ranges can change during continuous use of a column so that repeated calibration during quantitative studies is necessary. The different responses observed with the change of solid support are compared in Table I. Columns labeled A represent

TABLE I

VARIATION OF COLUMN EFFICIENCY AND RESPONSE WITH SOLID SUPPORT*

Compound	*Micrograms injected*		*Peaks specific response* ($cm^2/\mu g$)		*Relative response to cholestane*		*Theoretical plates*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Cholestane	0.6	0.3	4.30	4.33	1.00	1.00	930	2200
Cholestan-3β-ol	1.8	0.9	3.03	3.56	0.701	0.821	900	2700
Cholestane-3β,7α-diol	3.0	1.5	2.17	3.20	0.505	0.740	760	1760
Cholestane-3β,5α,6α-triol	15.0	7.5	0.61	3.18	0.140	0.735	1120	2500

* Figures in columns labeled A are from low efficiency columns (Chromosorb-W, 120–140 mesh); figures in columns labeled B are from high efficiency columns (Gas Chrom-P, 80–100 mesh).

the low efficiency support and B the high efficiency one. While the hydrocarbon cholestane is not affected by the change in support, the responses of more highly oxygenated compounds are moderately increased by the increasing column efficiency. The effect is most marked for cholestane-3β,5α,6α-triol which was in mid-linear range on one support and above linear range on the second support with an injection one half as large. The increased column efficiency made it difficult to define a linear portion of the dose–response curve for the oxygenated compounds. Calibration curves for

cholestan-3β-ol for the two supports are shown in Fig. 1. With the original support (Curve A) the linear range is relatively broad and easily distinguished while with the Gas Chrom-P (Curve B), the linear range is more difficult to define and the dose–response curve is paraboloid in shape. While sensitivity is increased accurate quantitation has become more difficult. These results are due to a combination of factors.

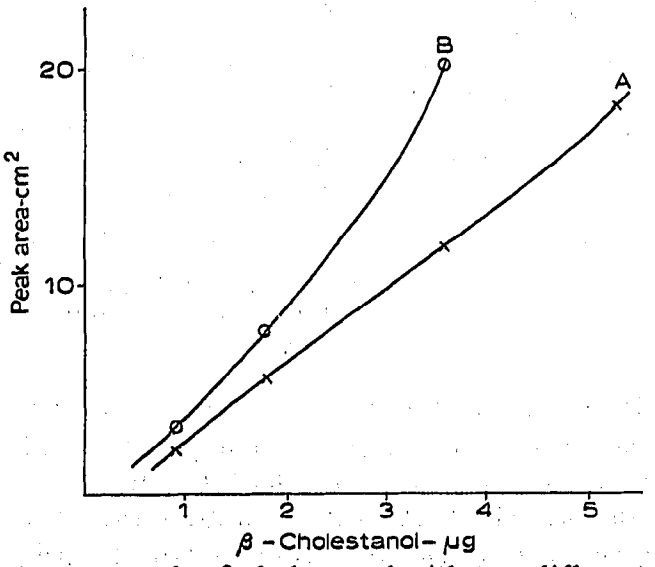

Fig. 1. Calibration curves for β-cholestanol with two different supports. See text.

First of all the basis for linearity of the argon instrument is that response is a function of both the total mass delivered and the rate at which the mass is being presented to the detector[7]. As the number of theoretical plates increases, sharper mass peaks are presented to the detector. Absolute threshold values diminish and detector over-load is experienced with correspondingly smaller amounts. A second factor which is responsible in part for the differences observed in Table I probably relates to the adsorption characteristics of the solid support. It may well be that the finer mesh Chromosorb-W was responsible for more adsorption and tailing than was the Gas Chrom-P although both supports were prepared in identical fashion.

Variation in column efficiency and detector response during continuous use

During a period when the Gas Chrom-P column was in continuous use quantitating fecal non-saponifiable material, it was observed, by comparing standards, that responses were changing gradually and these had to be meticulously taken into account in calculating the fecal sterols. Table II gives the data from two similar injections of the standard sterol mixture made 20 h apart. The changes seen in Table II are not due to any errors in injection technique since three standard injections were made each time. The differences in column detector performance obvious in Table II are two-fold. Each peak specific area (molar response) has increased from 38 to 72 %. The relative response of each peak to cholestane has increased significantly in each instance. Cholestanetriol is beyond linearity with the dose of 7.5 μg whereas in the earlier sample it was linear at the 10 μg level. Of interest is the fact that on the low efficiency column[4] 7.5 μg was barely over threshold. One may account for the differences of Table II by scrutiny of Fig. 2 which contains reproductions of the chro-

TABLE II

COMPARISON OF RESPONSE OF STANDARD STEROL MIXTURE BEFORE AND AFTER 20 HOURS CONTINUOUS USE

Sample number*	*Compound***	*Relative retention time*	*Microgram injected*	*Area (cm^2)*	*Area/unit weight*	*Relative response to cholestane*
2	A	1.00	0.4	1.07	2.67	1.00
	B	1.61	1.2	2.71	2.25	0.842
	C	2.50	2.0	3.76	1.88	0.704
	D	3.62	10.0	17.7	1.77	0.662
36	A	1.00	0.3	1.11	3.70	1.00
	B	1.64	0.9	2.89	3.22	0.870
	C	2.51	1.5	4.03	2.68	0.725
	D	3.62	7.5	22.8	3.04	0.822

* Sample 2 was 2.0 μl injection of standard mixture; sample 36 was 1.5 μl injection.
** A = cholestane; B = cholestan-3β-ol; C = cholestane-3β,7α-diol; D = cholestane-3β,5α,6α-triol.

matograms of the standards at the beginning of the series (A) and at the end (B). While the retention times can be superimposed, the peaks of the later chromatogram are sharper, higher, and narrower. Theoretical plates have been increased from an average of 1900 to 2300, despite the fact that chromatogram A is a smaller dose which should calculate to more theoretical plates, other factors being equal[6]. The effect, not apparent for cholestane, is more obvious as oxygen functions are added to the molecule. Note the gross loss of linearity represented by the two peaks of cholestane-3β,5α,6α-triol. The cholestanetriol peak in curve B is recorded at 35 % sensitivity. In other words, the rate at which molecules are being presented to the detector has increased. Since the machine is not truly linear to begin with, this change in column efficiency

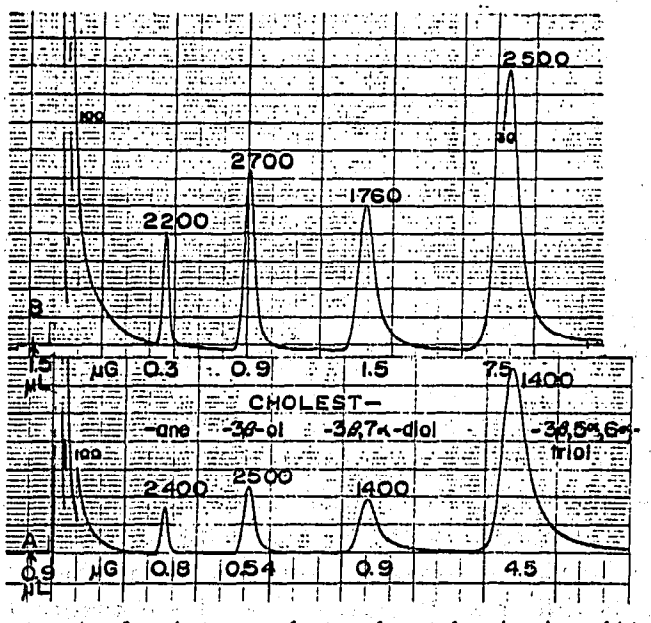

Fig. 2. Chromatograms of standard mixture of sterols at beginning (A), and end (B) of 20 h of fecal sterol quantitation. Theoretical plates for each peak are noted above, and dose size in micrograms is noted below each peak. The small numerals 30 and 100 are relative gains.

profoundly affects results in the same proportion as peak specific area was affected, *i.e.* 38–72 %. A second factor which may be responsible for the changes noted in Table II may be related to gradual filling of binding sites open on the adsorbent. These effects were noted despite routine loading doses[4] prior to quantitation.

Quantitation with high efficiency supports

The principles of quantitation of complex mixtures of fecal sterols are identical to those previously published[4]. Modification of the method is necessary to account for changing responses both with dose and time. Quantitation in this instance is done by running standards at regular intervals throughout the series of determinations and obtaining peak specific areas from one of two interpolations described below. Since the peak specific area is changing regularly as the column efficiency changes this may be plotted as shown in Fig. 3. Fig. 3 is plotted from data made from a series of 44 sample

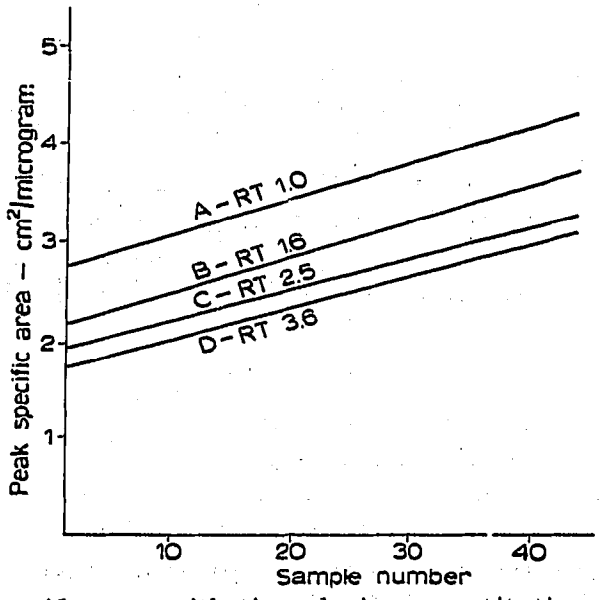

Fig. 3. Change of peak specific area with time during quantitative series. A = cholestane; B = cholestan-3β-ol; C = cholestane-3β,7α-diol; D = cholestane-3β,5α,6α-triol. RT = relative retention time to cholestane.

runs, each lasting about 30 min. Standards were run at intervals during the study. The responses of all compounds changed gradually with time. The lines are plotted by compound name and retention time so that unknown peaks at any time can be interpolated into the drawing on the basis of retention time[4] and sample number. Fig. 3 is satisfactory for quantitation when peaks are known to be in linear range, but this may be difficult to ascertain under the conditions described above.

The second method for determining peak specific areas of unknown peaks and one which takes the non-linearity and changing column efficiency into account is based on Figs. 3 and 4. Fig. 4 is a plot of relative responses to cholestane and peak height for each standard compound from the same series of determinations described for Fig. 3. It can be seen from Fig. 4 that relative responses increase gradually as larger amounts of standard are injected. This relationship holds well over a broad range of column efficiencies. The lines drawn in Fig. 4 are from an average of 12 points

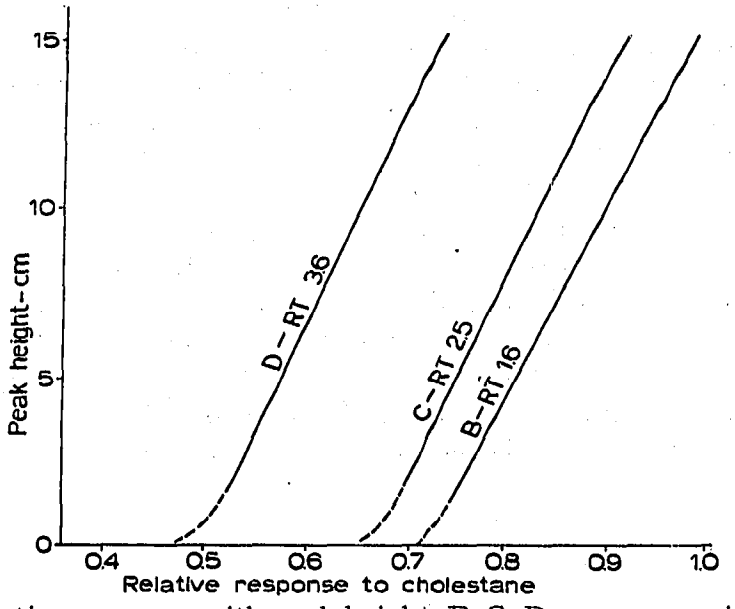

Fig. 4. Variation of relative response with peak height. B, C, D are same as in Fig. 3. RT = relative retention time to cholestane.

for each line. From Fig. 4 a line representing the relative retention time of any unknown may be interpolated visually and from the peak height of the unknown, a relative response is noted. The peak specific area of cholestane can be determined from Fig. 3 and the calculations made according to the formula:

$$m = \frac{A}{Sc \times RR} \qquad (1)$$

where: m = the weight of the unknown peak;
A = the area of the unknown peak;
Sc = the peak specific area of cholestane;
RR = the relative response of the unknown peak determined from Fig. 4.

This method of quantitation does not require an internal standard although when used it can be helpful. The internal standard is most useful when the proportion of cholesterol and coprostanol peaks is high with respect to other non-saponifiables, *i.e.* when the animal is fed a cholesterol enriched diet. Under these conditions a relatively large amount of internal standard may be added which will not be influenced significantly by small peaks in fecal non-saponifiable material with retention times equal to cholestane. When the cholesterol and coprostanol content of feces is low with respect to other non-saponifiable materials, small peaks may interfere with internal standardization and must be corrected for by two separate chromatograms, with and without the internal standard. Without the internal standard careful control of volumes is essential.

While the method outlined above seems laborious the advantages are great. A fractional analysis is helpful even though each component cannot always be identified. Plant sterols such as β-sitosterol which appear in many diets are separated from cholesterol and coprostanol so that the latter may be quantitated without interference. Table III is an example of the data obtained from balance studies in two groups of animals on high fat diets supplemented with 1% cholesterol. In group A the fat is butter in which the sterol is largely cholesterol and the cholesterol–coprostanol peaks

TABLE III

VALUE OF FRACTIONAL STEROL ANALYSIS BY GAS CHROMATOGRAPHY

Animal number	Diet*	Sterol per g rat feces measured by:				
		Gas chromatography				Digitonin precipitation
		Coprostanol	Cholesterol	Sitosterol**	Total	
1	A	11.2	40.7	<1	51.9	50.4
2	A	14.8	29.2	<1	44.0	47.4
3	A	4.4	48.7	<1	53.1	54.0
4	B	6.7	37.2	6.6	43.9	60.6
5	B	12.8	22.5	6.0	41.3	43.1
6	B	6.0	46.0	6.5	58.5	64.5

* Diet A contains 20% butter, 1% cholesterol; diet B contains 20% unsaturated margarine (Emdee Margarine, Pitman-Moore Co., Indianapolis 6, Ind.).

** Quantitation of sitosterol is only approximate since this method is based upon C-27 sterols and their oxygenated derivatives.

account for more than 95% of total fecal sterol under these conditions, closely agreeing with gravimetric data from digitonin precipitation. In group B, the fat is a highly unsaturated vegetable margarine with 0.7% content of sitosterols. When cholesterol and coprostanol peaks were analyzed, their sum was significantly less than the digitonin data indicated, the difference accounted for by fecal metabolites of the vegetable sterols. This information could not be appreciated in any study of fecal sterols by the Liebermann-Burchard or digitonin precipitation methods. Qualitative and quantitative differences such as cholesterol–coprostanol ratios[2] are also readily apparent. With improved column efficiency the more highly oxygenated compounds have better responses and can be more easily and accurately measured. The price for these results is meticulous control of quantitation with appropriate standards. It has not been my experience that "recalibration is not a serious problem" as claimed by ROSENFELD *et al.*[3] and repeated recalibration appears essential. Simple reliance upon linearity or "almost linear" can lead to errors as much as 100% in quantitative values. Since gas chromatography has the capacity for superior analysis of the complex sterols of feces it is important that the problems encountered with this method be clarified early before issues are clouded by conflicting results due to varied methodologies rather than varied biologies.

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SUMMARY

The argon ionization detector is not a truly linear instrument for the quantitative analysis of sterols. Because of this large changes in the molar response will occur when column efficiency is changed. These effects are described and methods are outlined for the quantitation of complex sterol mixtures under varying conditions. The necessity for careful control and standardization is emphasized.

REFERENCES

1 C. C. SWEELEY AND T. CHANG, *Anal. Chem.*, 33 (1961) 1860.
2 J. D. WILSON, *J. Lipid Res.*, 2 (1961) 350.
3 R. S. ROSENFELD, M. C. LEBEAU, S. SHULMAN AND J. SELTZER, *J. Chromatog.*, 7 (1962) 293.
4 D. K. BLOOMFIELD, *Anal. Chem.*, 34 (1962) 737.
5 E. C. HORNING, E. A. MOSCATELLI, C. C. SWEELEY, *Chem. & Ind. (London)*, (1959) 751.
6 C. J. HARDY AND F. H. POLLARD, *J. Chromatog.*, 2 (1959) 1.
7 J. E. LOVELOCK, *J. Chromatog.*, 1 (1958) 35.