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THE SEPARATION OF SOME VOLATILE FATTY ACIDS ON A "SEPHADEX" PARTITION CHROMATOGRAM

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ABSTRACT

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The suitability of Sephadex LH-20 as a solid support for the partition chromatography of acetic, propionic and butyric acids was investigated by using standard mixtures of ^{14}C-labelled acids. The clear separation that was obtained between the acids was confirmed by the negligible cross contamination found between the acid peaks. Furthermore, the Sephadex LH-20 column gave a convenient elution volume and time similar to those found for a Celite Analytical Filter Aid column, without the progressive deterioration in separation normally associated with Celite.

Résumé

LA SÉPARATION DE QUELQUES ACIDES VOLATILES GRAS PAR LA CHROMATOGRAPHIE DE PARTAGE À SÉPHADEX

En employant des mélanges normaux d'acides marqués à ^{14}C, les auteurs ont recherché l'utilité du Séphadex LH-20 comme support stable dans la chromatographie de partage des acides acétique, propionique et butyrique. La contamination croisée entre les sommets des acides a été minimale et prouve que la séparation realisée a été nette. En plus, la colonne de Séphadex LH-20 a fourni un volume et un temps de séparation assez proches de ceux obtenus avec la colonne Celite Analytical Filter Aid, mais sans la détérioration progressive dans la séparation que l'on rencontre assez souvent avec cette dernière.

INTRODUCTION

Silicic acid has been widely and successfully used as a solid support for partition chromatography of the volatile fatty acids (VFA) by, among others, Smith (1942), Elsden (1946), Ramsey (1963) and Leng & Leonard (1965). Celite, a diatomaceous earth with properties similar to those of silicic acid, was used by Petersen & Johnson (1948), Gray, Pilgrim & Weller (1951) and Wiseman & Irwin (1957) in order to avoid the difficulties inherent in the preparation of silica gel. Furthermore, the Celite required smaller, more convenient elution volumes than the silicic acid. Therefore, a method based on the above-mentioned techniques was used to separate the VFA extracted from rumen fluid (Van der Walt & Briel, 1976).

Values for the interconversion of propionic to butyric acid in the rumen, obtained by this method of separation, were higher than those reported by other workers under similar experimental conditions (Leng & Leonard, 1965; Bergman, Reid, Murray, Brockway & Whitelaw, 1965). This pointed to the fact that, despite the apparently clear visual separation of the bands eluted from the column, contamination of the butyric fraction by propionic acid had occurred. Furthermore, the separation efficiency of the Celite column was found to deteriorate progressively with use.

Sephadex LH-20+, a bead-formed, hydroxy-propylated dextran gel, having both hydrophilic and hydrophobic properties, has been successfully used in "straight-phase" partition chromatography because of its ability to take up selectively the polar solvent and thus create an extremely stable, high volume stationary phase (Anon., 1973). The present investigation showed that Sephadex LH-20 provided a suitable solid support for the efficient separation of acetic, propionic and butyric acids with negligible cross contamination between the peaks.

MATERIALS AND METHODS

Reagents

Chemicals: Unless otherwise specified in the text, all chemicals were obtained from Merck, Darmstadt and were of analytical grade. A set of solvents having increasing polarity (2%; 7,5% and 15% 1-butanol in benzene, v/v and referred to as BB_2, $BB_{7,5}$ and BB_{15}) was used to elute the VFA from the columns. All these elution solvents were equilibrated against water before use and filtered through Whatman PS 1 paper* to remove any suspended water droplets.

Standard solution: A standard VFA solution was prepared by dissolving acetic (43,1 meq/l), propionic (16,4 meq/l and butyric acid (13,6 meq/l) in wet $BB_{7,5}$. This standard resembled the final composition of the VFA samples extracted from ruminal fluid of sheep fed lucerne hay (Van der Walt & Briel, 1976).

Radiolabelled mixtures: Two radiolabelled VFA mixtures were used to establish the extent of the cross-contamination occurring between the acids during elution. One, the A & B standard, contained sodium-1-^{14}C acetate** (27 uCi/1) plus sodium-1-^{14}C butyrate (21 uCi/1) dissolved in the standard VFA solution described above; while the other, the P standard, contained sodium-2-^{14}C propionate** (21 uCi/1).

Column preparation: Preparation of the Sephadex column incorporated several suggestions from a Pharmacia handbook (Anon., 1973). Six grammes (dry mass) of Sephadex LH-20 beads were allowed to swell in 30 ml of an 0,05% (m/v) aqueous solution of alphamide red-R*** for 24 h. The resultant slurry was poured into a glass column (350×12 mm) fitted with a No. 0 sintered glass filter disc above the outlet. The bed was evenly packed by allowing the excess dye solution to flow through the column under gravity soon after the slurry had been transferred. The aqueous dye solution filling the void volume was replaced by wet BB_{15} under a pressure of 10 kPa, and the packing was thereby further settled. This rinsing was continued until a 2 ml aliquot of effluent required less than 0,4 ml 0,005 N sodium hydroxide for neutralization. The column was equilibrated against 20 ml BB_2 prior to the application of a sample and stored between analyses in this condition.

+ Pharmacia Fine Chemicals AB, Uppsala, Sweden

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** Radiochemical Centre, Amersham, England

*** Kanto Chemical Co., Tokyo, Japan

Column development

The procedure previously used for elution through a Celite Analytical Filter Aid column (Van der Walt & Briel, 1976) was adapted as follows: A 1 ml aliquot of a standard mixture was allowed to drain into the Sephadex LH-20 column under gravity, and then rinsed further into the packing with 1 ml of BB_2. Subsequent development of the column was carried out under a pressure (*ca.* 10 kPa) sufficient to maintain an elution rate of *ca.*1 ml/min. The butyric acid component was brought to the bottom of the column using the least polar solvent BB_2, while the propionic and acetic acid bands were eluted with $BB_{7,5}$ and BB_{15}, respectively. Convenient aliquots (usually 2 ml) of effluent were collected, equilibrated with 20 ml water under a nitrogen atmosphere for 3 min and titrated against 0,005 N sodium hydroxide to an end-point of pH 9 using a Metrohm Combititrator*.

Estimation of radioactivity

When the labelled standard mixtures were used, suitable fractions of the eluent, representative of each component, were combined, taken to pH 11, dried, transferred by 5×1 ml water rinses to liquid scintillation vials containing 10 ml Instagel**, and the radioactivity determined in a Packard Liquid Scintillation Spectrometer Series 3 000**. The channels ratio method of quench correction was used to convert the data to absolute activity.

* Metrohm, Herisau, Switzerland

** Packard Instruments

RESULTS

A typical separation pattern of the acetic, propionic and butyric acids in the standard VFA solution after elution through the Sephadex column is shown in Fig. 1. Appropriate blank corrections, obtained from titres of equal volumes of eluent passed through a blank column under similar conditions, were subtracted to obtain this histogram. The clear separation of the components of the standard solution confirmed the visual separation of the bands as they passed down the column. The butyric acid component required 20 ml of the least polar solvent BB_2, propionic acid 20 ml of $BB_{7,5}$ and acetic acid 40 ml of the BB_{15} solvent mixture for elution.

Recovery of both labelled VFA mixtures separated on the column is shown in Table 1. Although relatively large fractions were collected (2 ml), thus making calculation less precise, the results obtained from 2 separate columns indicate that there is little loss of acid in transit through the column.

The possible contamination of the propionic acid peak by acetic and butyric acids was determined by the elution of a 1 ml aliquot of the A+B labelled standard. The fractions indicated in Fig. 1 yielded the specific activities for each peak listed in Table 2a. It can be seen that the propionic acid component contained insignificant amounts of either butyric or acetic acid. Similarly, the elution of 1 ml of the P labelled standard revealed negligible contamination of the butyric acid component by propionic acid (Table 2b). All of the labelled VFA showed the same specific activity before and after elution thereby indicating that no dilution by adjacent unlabelled VFA was taking place.

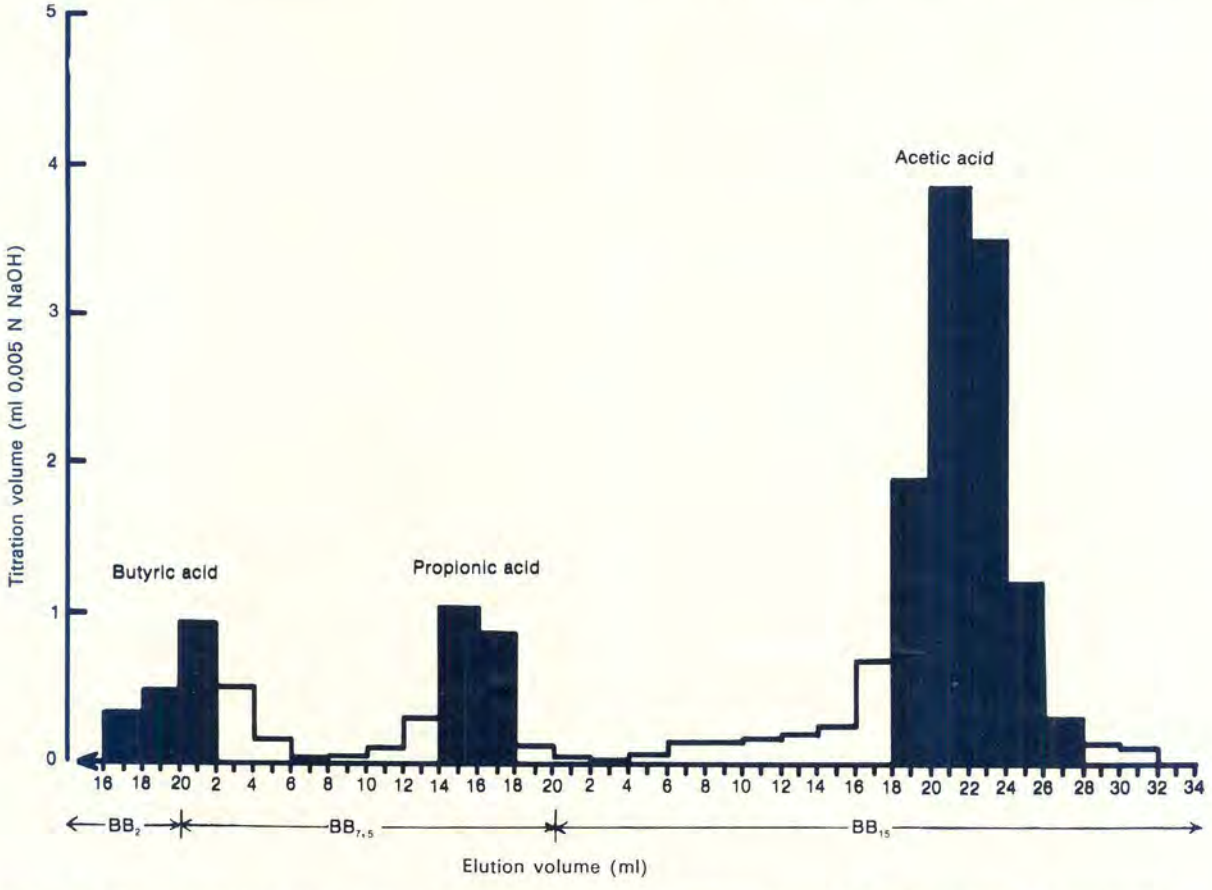

FIG. 1 A typical separation of a standard VFA mixture obtained through a Sephadex LH-20 column The fractions indicated by the black areas were combined for the radioactivity measurements listed in Table 2

TABLE 1 Total recovery values obtained by elution of a 1 ml aliquot through a Sephadex LH-20 column after pooling the fractions containing the peaks

VFA	Amount added	Amount recovered					
		Column 1		Column 2		Average	
	meq/l	meq/l	%	meq/l	%	meq/l	%
Acetic	43,1	42,6	99,5	44,8	104,7	43,7	102,1
Propionic	16,4	14,9	91,8	16,1	98,9	15,5	95,4
Butyric	13,6	12,7	94,1	12,7	94,1	12,7	94,1
Total	73,1	70,2	96,8	73,6	101,4	71,9	98,3

TABLE 2 (a) A 1 ml aliquot of the standard mixture (A+B) containing 626 nCi/meq acetic acid and 1 544 nCi/meq butyric acid was eluted through a Sephadez LH-20 column and the pooled fractions shown in Fig. 1 used for the specific activity determination

Column	Specific activity recovered						
	Butyric acid (B)		Propionic acid (P)			Acetic acid (A)	
	nCi/meq	%	nCi/meq	as % of B	as % of A	nCi/meq	%
1	1 513	98,0	5,0	0,3	0,8	652	104,2
2	1 585	102,7	10,6	0,7	1,7	590	94,3
3	1 545	100,0	8,9	0,6	1,4	595	95,1
Average	1 548±36	100,3	8,2	0,5	1,3	612±34	97,8

(b) A 1 ml aliquot of the standard mixture (P) containing 1 280 nCi/meq propionic acid yielded the following results when analysed as above

Column	Specific activity recovered					
	Butyric acid		Propionic acid		Acetic acid	
	nCi/meq	%	nCi/meq	%	nCi/meq	%
1	3,62	0,3	1 246	97,3	3,09	0,3
2	0,54	0,0	1 254	98,0	5,68	0,5
3	1,79	0,2	1 387	108,4	1,92	0,2
Average	1,98	0,15	1 296±79	101,2	3,56	0,3

Discussion

The solvent system required to elute the VFA from a Sephadex LH-20 column (BB_2–BB_{15}) was considerably more polar than that necessary for the Celite Analytical Filter Aid support (BB_0–$BB_{7.5}$, Van der Walt & Briel, 1976), thus indicating a parallel difference in polarity between the respective stationary phases. All standard VFA mixtures, as well as samples previously analysed (Van der Walt & Briel, 1976) were dissolved in $BB_{7.5}$ as recommended by Gray, Pilgrim & Weller (1951). Therefore, the addition of the 1 ml sample to the Celite column could have created a composite solvent front that contained sufficient 1-butanol to dislodge *ca.* 10% of the propionic acid (Van der Walt, unpublished observations, 1975) adsorbed to the weakly polar solid support and elute this together with the butyric acid component. The more polar Sephadex LH-20 packing was less influenced by this composite solvent front despite the slightly higher polarity involved (BB_2+$BB_{7.5}$) as shown by the neligible carry-over of propionic into butyric acid found (0,15%).

Decomposition of the indicator, rhodamine red-R, was noted after some time in the case of both the Celite and Sephadex solid supports, although the indicator appeared to be more stable on the former. This phenomenon which had been previously reported by Wiseman & Irvin (1957) and Wiseman, Stone, Savage & Moore (1952), posed no problem in this laboratory as each column was precisely calibrated against labelled standards on a volume basis using the indicator merely as an initial guide. As acetone has been shown to play a protective role (Wiseman & Irvin, 1957), the problem may be overcome by developing another solvent system for the elution of the VFA consisting of a series of hexane and acetone mixtures prepared in similar fashion to the benzene and 1-butanol mixture

described above. Alternatively, the rhodamine red-R indicator may be substituted by Moir's modified methyl orange which does not seem to be as prone to decomposition (Gray *et al.*, 1951).

In order to prevent elution of the indicator by the organic solvents, Wiseman & Irwin (1957) used sucrose to stabilize the Celite Analytical Filter Aid stationary phase. The Sephadex LH-20 column required no sucrose by comparison, thereby further emphasizing the greater stability of this support material.

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