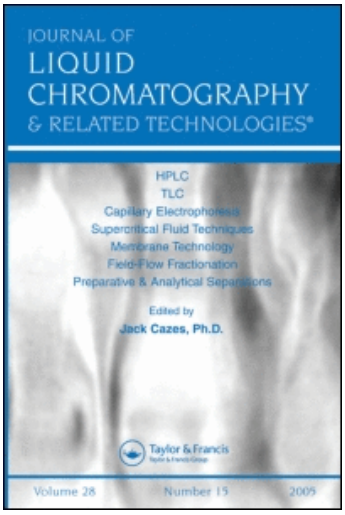

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Avery, N. C.(1996) 'The Use of Solid Phase Extraction Cartridges as a Pre-Fractionation Step in the Quantisation of Intermolecular Collagen Crosslinks and Advanced Glycation End-Products', Journal of Liquid Chromatography & Related Technologies, 19: 11, 1831 — 1848
To link to this Article: DOI: 10.1080/10826079608014009
URL: http://dx.doi.org/10.1080/10826079608014009

J. LIQ. CHROM. & REL. TECHNOL., 19(11), 1831–1848 (1996)

THE USE OF SOLID PHASE EXTRACTION CARTRIDGES AS A PRE-FRACTIONATION STEP IN THE QUANTITATION OF INTERMOLECULAR COLLAGEN CROSSLINKS AND ADVANCED GLYCATION END-PRODUCTS

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ABSTRACT

A range of solid phase extraction (SPE) sorbents was investigated to determine their suitability as a pre-fractionation step during the quantitation of collagen crosslinks and advanced glycation end products by reverse phase high performance liquid chromatography (RP HPLC).

Propylsulphonic acid (PRS) SPE cartridges were shown to be the most suitable sorbent type by testing for resolution of specific amino acids using both inorganic salt buffers and volatile organic solvents.

The loading capacity of the PRS-SPE cartridges was determined and the efficacy of the cartridges evaluated by comparison with previously published methods using fibrous cellulose and SP Sepharose.

PRS-SPE cartridges provided a superior, rapid, cost effective alternative to either of these pre-fractionation steps giving a higher recovery of both the mature collagen crosslink, pyridinoline, and the advanced glycation end-product crosslink, pentosidine.

INTRODUCTION

Collagen is stabilised by intermolecular crosslinks formed following the enzymic oxidation of specific lysines in the collagen molecule by lysyl oxidase. The initial products are intermediate divalent crosslinks that, with time, are spontaneously converted into trivalent mature crosslinks. {For a review see Bailey.}[1] Quantitation of the intermediate collagen crosslinks dehydro-hydroxylysinonorleucine (Δ-HLNL), hydroxylysinoketo-norleucine (HLKNL) and dehydro-histidinohydroxymerodesmosine (Δ-HHMD) requires their reduction with sodium borohydride to stabilise them prior to± acid hydrolysis. The trivalent, mature collagen crosslinks hydroxylysyl pyridinoline (Hyl-Pyr), lysyl pyridinoline (Lys-Pyr) and histidinohydroxylysinonorleucine (HHL) are all stable to acid hydrolysis without prior reduction.

It is possible for all these crosslinks to be quantified from an acid hydrolysate of reduced, collagenous tissue by ion-exchange chromatography using either ninhydrin or another post-column detection system. Alternatively pyridinoline, being naturally fluorescent, is commonly quantified by exploiting this characteristic using a reverse phase high performance liquid chromatography (RP HPLC) procedure.[2]

Collagen has a long biological half-life and adventitiously accretes glucose by non-enzymic processes. The initial product of this reaction can in turn be oxidised to form several compounds referred to as advanced glycation end-products (AGE's), some of which act as intermolecular crosslinks. {For a review see Paul and Bailey}.[3]

One such crosslinking component has been characterised and named pentosidine, based on its proposed derivation from ribose. Like pyridinoline, pentosidine is naturally fluorescent and can also be quantified by RP HPLC using fluorescence detection.[4]

Due to the paucity of all these crosslinking components in most tissue hydrolysates, it is frequently necessary to undertake a pre-fractionation step to enhance their relative concentrations over the other constituent amino acids. In 1988, Black et al[5] reported a pre-fractionation step using fibrous cellulose (CF1) to concentrate pyridinoline.

This procedure was subsequently modified and shown by Sims and Bailey, in 1992[6], to also be suitable for concentrating both the intermediate and mature collagen crosslinks. However, in our experience CF1 pre-fractionation of hydrolysates for pentosidine quantitation is not reliable since pentosidine is desorbed to a variable degree by the organic eluant.

This variability can be controlled, but not eliminated, by keeping both loading volume and organic wash volume as small as practically possible. In practice this means loading samples in less than 30% of the column volume and limiting the organic washes to no more than 5 column volumes. In this fashion it is possible to keep the losses of pentosidine to considerably less than the 88% reported by Takahashi et al,[7] but is none the less still unacceptably high.

Sell and Monnier[8] reported using sulphopropyl (SP) Sephadex cation exchange columns to purify their fluorescent glycation components, whilst Dyer et al.[9] employed a C_{18} Sep pack cartridge as a pre-fractionation step to concentrate MFP-1, shown to be pentosidine in that same paper.

Takahashi et al.[7] reported better than 70% recovery of pentosidine and pyridinoline standards using the same SP Sephadex cation exchange sorbent compared to better than 70% recovery of pyridinoline but only 12% recovery of pentosidine from CF1

In this paper, we report the results of a detailed survey of normal and reversed phase, solid phase extraction (SPE) cartridges, the selection and testing of propylsulphonic acid (PRS) SPE cartridges and the development of a rapid, simple method for the recovery of both mature collagen crosslinks and AGE standards in good yield using volatile organic buffers.

Finally, we report the results of a comparison of PRS solid phase extraction with CF1 pre-fractionation of twelve whole muscle hydrolysates preceding collagen crosslink quantitation by both amino acid analysis and RP HPLC. We had hypothesised, that variation in muscle growth rate (as indicated by their growth coefficients), might be reflected in variation in the intra-muscular collagen crosslinking and as muscle has such a low collagen content, it provided a rigorous test material for comparison between the established methodologies and the proposed new method.

Table 1

Selected Muscles and their Reported Growth Coefficients

Muscle Name	Growth Coefficient	Relative Growth Group
Gastrocnemius	0.84*	Early
Extensor carpi radialis	0.85	Early
Flexor hallucis longus	0.86	Early
Semitendinosus	0.91*	Average
Psoas major	0.92*	Average
Supraspinatus	0.94	Average
Triceps brachii	0.99	Average
Pectoralis profundus	1.00	Average
Latissimus dorsi	1.05	Late
Semispinalis capitis	1.07*	Late
Serratus ventralis thoracis	1.13	Late
Splenius	1.18	Late

* Averaged value for Multiphasic growth pattern.
Data from Butterfield & Berg.[10]

MATERIALS AND METHODS

Reagent.

Unless otherwise stated, all reagents were Analar grade purchased from Fisons Chemicals (Leicestershire, England). HPLC solvents were purchased from Rathburn Fine Chemicals (Peebleshire, Scotland). Amino acid standards were purchased from Pierce Warriner (Cheshire, England) and crosslink standards were isolated and purified in our own laboratory.

Meat Sample.

A Charolais x Freisian-Holstein steer, 25 months old, was purchased locally and slaughtered under humane, hygienic conditions in the Bristol University abattoir. The carcass was left to hang at ambient temperature (>10°C) overnight, before removal to a chiller for a further 24 hours.

Twelve muscles were selected (Table 1) which, according to their reported growth coefficients,[10] could be categorised as having early, average or late relative growth development and were removed from one side of the carcass.

Samples were processed as described by Avery and Bailey,[11] in brief all the epimysium and associated fat was removed from each of the muscles which were then cut into manageable cubes and separately comminuted in a Moulinette S (Moulinex, France) food processor. 1g aliquots of each muscle were suspended in phosphate buffered saline (PBS) at pH 7 and the intermediate crosslinks stabilised by reduction with nascent hydrogen released from sodium borohydride over a 2 hour period at room temperature.[12]

After pH adjustment to stop the reaction, the samples were washed with distilled water (to remove salt) filtered, freeze-dried, weighed and powdered in the food processor, prior to weighed aliquots (100mg) being hydrolysed in 15mL of constant boiling hydrochloric acid (HCl) at 112°C for 24 hours in hydrolysis tubes sealed after gassing with nitrogen.

SPE Cartridge Type Selection.

100mg, Bond Elut SPE cartridges (Analytichem International Inc. California, USA) of twelve different sorbent types (Table 2), were washed, pre-conditioned and equilibrated in a solvent most suited for analyte binding, according to the information supplied by the manufacturer.

A standard solution of hydroxyproline and lysine (10 mg/mL) in 0.01M HCl, was prepared and 10µl loaded on to each cartridge type. These amino acids were selected to represent the range of pKa characteristics of the majority of amino acids, including the collagen crosslinks, present in a collagenous tissue hydrolysate. Also, hydroxyproline produces a different coloured product when reacted with ninhydrin, making resolution of the two more readily detectable. The loading volume was kept small to minimise cartridge re-conditioning by the 0.01M HCl.

10 bed volumes (1mL) of the equilibration solvent were passed through each cartridge to determine how strongly bound the amino acids were, followed by 10 bed volumes of the suggested elution solvent for each sorbent type. After each bed volume, a 10µl drop of the eluant was dried onto filter paper and sprayed with a 0.2% solution of ninhydrin in acetone and heated at 100°C for 2 minutes to detect the colour of the eluted amino acids.

Table 2

Relative Retention of the Amino Acid Test Mixture (Hydroxyproline & Lysine) by SPE Sorbents

Matrix Type	Elution Position in Equilibrium		Or Eluting Solvent	
	First 50%	Final 50%	First 50%	Final 50%
Non-polar				
Octadecyl	+++	++		
Octyl	++++	+		
Ethyl	+++++			
Cyclohexyl	+++++			
Phenyl	+++++			
Polar				
Cyanopropyl	+++++			
Diol	+++++			
Aminopropyl	+++++			
Cation Exchange				
SCX		++++	+	
CBA	++	+++		
Anion Exchange				
SAX	+++++			

Cationic SPE Cartridge Selection

Three types of cation exchange SPE cartridges were obtained from three different manufacturers. (i) CBA cartridges possess a weakly ionic, carboxymethyl group, (ii) PRS cartridges possess a sulphonylpropyl group and are intermediary in ionic strength between CBA and (iii) SCX cartridges which possess a benzenesulphonylpropyl group and are strongly ionic. A range of inorganic buffers of different pH's was used to resolve the same two standard amino acids. The recovery of the hydroxyproline standard from each manufacturers sorbent type was precisely quantified by means of a ChemLab Autoanalyser (Chemlab Instruments Ltd, Essex, England) utilising the method of Bannister and Burns.[13]

The cartridges were prepared as before according to the manufacturers instructions then equilibrated in 0.1M citrate buffer pH 2.1. The sample was loaded in 10µl of 0.01M HCl and eluted with a succession of 1mL washes of

0.1M citrate buffered at pHs 2.1, 3.1, 3.5, 4.7, 6.5 and 8.5 and finally with 4N ammonium hydroxide to displace any residual material. All washes were collected and the recovery of hydroxyproline determined as described above. In addition, 10µl aliquots were spotted on to filter paper and sprayed with ninhydrin, as before, to determine the elution position of lysine.

SP Sepharose Pre-fractionation v. PRS Solid Phase Extraction Cartridges

PRS solid phase extraction cartridges were used to test a standard mixture of the fluorescent, mature collagen crosslink pyridinoline and hydroxyproline. This mixture, also in 0.01M HCl, was loaded on to both 500mg and 1g PRS cartridges (International Sorbent Technology, Glamorgan, Wales) packed in 3mL syringe bodies, to prohibit elution variation due to changes in cartridge dimension, and eluted as before with the citrate buffers. An equal amount of the same standard mixture was loaded on to 4mL and 8mL columns of SP Sepharose (Pharmacia, Buckinghamshire, England) prepared in the laboratory. These Sepharose columns were eluted with 0.15M and 1M HCl, according to the method of Takahashi et al.[7]

All eluants were collected, vacuum concentrated and the recoveries of all components calculated as a percentage of the initial loading. Pyridinoline was quantified by RP HPLC using a Hypercarb S, (Shandon HPLC, Cheshire, England) graphitic carbon column, 100 x 4.6mm, with a gradient from 0-12% tetrahydrofuran in water, each containing 0.5% trifluoroacetic acid and a flow rate of 1mL / minute. The pyridinoline standard was detected by means of its natural fluorescence using an LS5 luminescence spectrophotometer (Perkin-Elmer, Buckinghamshire, England.), with excitation set at 295 nm and emission at 405 nm. Bailey et al.[14]

PRS-SPE Cartridge Preparation and Eluting Buffer Modifications.

Modifications were made to both the cartridge manufacturers preparation procedure and the elution buffers. The initial cartridge "wetting" with methanol, was modified by incorporating 1% triethylamine in the methanol and increasing the wash volume to 10 cartridge volumes. The methanol was displaced with 3 volumes of water containing 10% methanol followed by 3 volumes of 0.1M HCl and finally equilibrated with 3 volumes of loading buffer also containing 10% methanol. The inclusion of methanol was intended to prohibit hydrophobic interactions with the propyl chain of the propylsulphonic acid functional group. A selection of organic volatile buffers (Table 5) was prepared according to published data,[15] covering the same pH range as the citrate buffers and each containing 10% methanol.

PRS Cartridge Loading Capacity

To determine the loading capacity of both the 500mg and 1g PRS cartridges, a known dry weight of bovine serum albumen (BSA) was hydrolysed, then freeze-dried and rehydrated in the volatile loading buffer. A constant volume but variable weight of BSA was added to a constant amount of both standard fluorescent crosslinks, pyridinoline and pentosidine, plus hydroxyproline.

A range from 1 to 15% of the cartridge sorbent dry weight of BSA was loaded on both PRS cartridge sizes and the components eluted using the new volatile buffers. The cartridge eluant at each pH was analysed for hydroxyproline and fluorescent crosslink recovery after vacuum concentration as described above.

CF1 Pre-fractionation v. PRS Solid Phase Extraction Cartridges

After drying and re-hydrating in the respective equilibration solvents, aliquots from the twelve reduced, acid hydrolysed, whole bovine muscles, equal to 30mg, were loaded on to 3 mL CF1 columns, prepared in the laboratory, and 1g PRS-SPE cartridges. The aqueous eluant from the CF1 and the combined pH 3.5 and 4.7 eluants from the SPE cartridges were dried down separately and analysed, according to the method of Sims and Bailey,[6] using an Alpha Plus amino acid analyser (Pharmacia, Buckinghamshire, England). Aliquots of the same eluants from duplicate pre-fractionations were analysed by RP HPLC as previously described.[14]

RESULTS

SPE Cartridge Type Selection

Table 2 shows the relative retention of hydroxyproline and lysine by the 12 different sorbent types from Analytichem International Inc. No resolution of hydroxyproline from lysine by any of the matrices was achieved, as judged by colour development with ninhydrin, all spots appearing blue/grey. However, on the basis of the retention of both the test amino acids until late in the equilibration washes, it was judged worthwhile to undertake a further investigation of the available range of cation exchange SPE cartridges.

Table 3

% Recovery of Hydroxyproline from Cation SPE Sorbents

	Manufacturer 1			Manufacturer 2		Manufacturer 3	
Eluant	**CBA**	**PRS**	**SCX**	**CBA**	**SCX**	**CBA**	**SCX**
Load (pH2.1)	102*	102	0	104*	0	98*	0
pH3.1	0	4.4	0	0	0	0	0
pH3.5	0	3.7	0	0	0	0	0
pH4.7	0	0*	0	0	0	0	0
pH6.5	0	0*	0	0	0	0	0
pH8.5	0	0	0	0	0	0	0
4N	0	0	52.1	0	102.8	0	38.9

* Indicates the elution position of the lysine, i.e. during loading or elution, as judged by ninhydrin spraying.

Cationic SPE Cartridge Selection

Table 3 illustrates the binding strength of the amino acid test mixture to each cationic cartridge type and shows the percent recovery of hydroxyproline from the three different types of SPE cation sorbent.

These results demonstrate that the strong cation exchange (SCX) cartridges bound the standard amino acids too tightly, requiring high salt concentrations to desorb them, which, would result in subsequent problems for amino acid analysis. Conversely, the CBA cartridges failed to bind either standard amino acid effectively. The apparent resolution of lysine and hydroxyproline by the PRS solid phase extraction cartridges suggested that further investigation of these cartridges was worthwhile, however, the elution of very small amounts of hydroxyproline from the same cartridges at higher pHs ("ghosting"), suggested some form of non-specific binding was occurring.

Table 4

% Hydroxyproline and Pyridinoline Recovery from PRS Cartridges v. that from SP Sepharose Columns

	500 mg PRS		1g PRS		4 mL Sepharose		8mL Sepharose	
Eluant	**Hyp**	**X-Lnk**	**Hyp**	**X-Lnk**	**Hyp**	**X-Lnk**	**Hyp**	**X-Lnk**
pH2.1	36	0	0	0				
pH3.1	59	0	0	0				
pH3.5	0	0	92	0				
pH4.7	0	86	0	104				
0.15M					60	0	59	0
1.0M					11	78	16	69

(Values are the average of triplicate loadings.)

SP Sepharose Pre-fractionation v. PRS Solid Phase Extraction Cartridges

Table 4 shows the hydroxyproline and fluorescent crosslink recovery from the PRS cartridges using citrate buffers and from the SP Sepharose columns using dilute HCl. Both the hydroxyproline and pyridinoline recovery from the PRS cartridges, is greater than that from the SP Sepharose columns and the resolution between these standards is more pronounced. In addition, not all the standard mixture seems to have desorbed from the SP Sepharose and continued elution with HCl failed to desorb them. The volume of eluant involved for PRS desorption is significantly smaller than that for SP Sepharose, resulting in considerably shorter run times and faster subsequent processing by freeze-drying or vacuum concentrating.

The recoveries of both pyridinoline and hydroxyproline, from the Sepharose columns, are comparable to each other and similar to those reported by Takahashi et al.[7] for pentosidine and pyridinoline.

During subsequent analysis of these samples several disadvantages of the SPE procedure became apparent: (i) The manufacturers guidelines for cartridge preparation were inadequate, as some components were eluting from the cartridges, especially at high pH, and binding to the HPLC column causing severe retention time variation. (ii) The citrate residues after vacuum concentration were sufficiently large to cause problems for any subsequent amino acid analysis. (iii) The fact that hydroxyproline appeared to "ghost"

Table 5

Volatile Buffer Formulations for PRS Solid Phase Extraction Cartridges

Type / pH of buffer	Components
Washing / "wetting	1% Triethylamine in Methanol
Conditioning I	10% methanol in water
Conditioning II	10% methanol in 0.1N Hcl
Loading / Equilibration (pH 1.9)	87mL glacial acetic acid, 25mL 90% formic acid, 100mL methanol to 1litre
Eluting buffers	
pH 3.1	5mL pyridine, 100mL glacial acetic acid, 100mL methanol to 1L
pH 3.5	5mL pyridine, 50ml glacial acetic acid, 100mL methanol to 1L
pH 4.7	25mL pyridine, 25mL glacial acetic acid, 100mL methanol to 1L
pH 6.5	100 mL pyridine, 4mL glacial acetic acid, 100mL methanol to 1L

(Data, with modification, from Dawson et al.[15]

from both silicon and Sepharose matrices of the propylsulphonic acid functional group suggested that hydrophobic interactions with the propyl chain were responsible, rather than polar interactions with uncapped silanol groups. This led us to modify the cartridge preparation procedure and the eluting buffers.

PRS-SPE Cartridge Preparation and Eluting Buffer Modifications

See Table 5 for volatile buffer formulations. After thoroughly washing and conditioning the cartridges, care was taken to prevent drying out until after the sample was loaded, as recommended by the manufacturers. However, it was found that separation was enhanced if all of the previous eluant was removed before adding the next, i.e. if the cartridge was momentarily dried between buffers. The volume of new buffer required to change the pH of the cartridge effluent was found to be 2.8mLs for the 500mg and 4.6mLs for the 1g cartridge. Consequently 3mLs and 5mLs were used as working column volumes, respectively, for each cartridge size.

Table 6A

% Hydroxyproline Recovery from PRS-SPE Cartridges v. Loading

Cartridge Size	% of Sorbent Dry Weight Loaded	% Recovery in Each Eluting Buffer pH 1.9	pH 3.1	pH 3.5
500mg	1	0	81	27.5
500mg	3	42.5	57.5	
500mg	5	75	22	
500mg	10	90	12	
500mg	15	90	8	
1g	2	29	67	
1g	4	89	16	
1g	6	95	11	
1g	12	93	5	

(Values are the average of duplicate loadings

Table 6B

% Fluorescent Crosslink Recovery from PRS-SPE Cartridges vs Loading

Cartridge Size	% of Sorbent Dry Weight Loaded	Eluting pH	% Pyridinoline	% Pentoside
500mg	1	4.7	100	100
500mg	3	4.7	100	100
500mg	5	3.5	36	18
		4.7	72	85
500mg	10	3.5	50	11
		4.7	50	89
1g	2	4.7	100	100
1g	4	3.5	33	0
		4.7	67	100
1g	6	3.5	43	6
		4.7	56	94

(Values are the average of duplicate loadings.)

Table 7

Comparison of Intermediate and Mature Collagen Crosslink Quantitation in the Selected Muscles, Expressed as Moles Crosslink / Mole Collagen, following CF1 or PRS-SPE Cartridge Pre-fractionation and Quantitation by either Amino Acid Analysis of RP-HPLC

		Mature X-Link		Intermediate X-Links	
Muscle	**Pre-fract.**	**Pyr. (AAA)**	**Pyr. (RP-HPLC)**	**DHLNL**	**HLNL**
Gastrocnemium	CF1	0.12		0.08	0.07
	SPE	0.23	0.14	0.11	0.06
Ext. carp. rad.	CF1	0.13		0.07	0.03
	SPE	0.13	0.13	0.09	0.06
Flex. hal. long.	CF1	0.15		0.04	0.04
	SPE	0.08	0.16	0.02	0.03
Semitend.	CF1	0.09		0.30	0.70
	SPE	0.16	0.11	0.07	0.60
Psoas maj.	CF1	ND		0.16	0.09
	SPE	0.47	0.02	0.14	0.08
Supraspinatus	SPE	0.16		0.12	0.04
	CF1	0.20	0.15	0.08	0.04
Triceps. brach.	CF1	0.13		0.07	0.06
	SPE	0.26	0.14	0.11	0.05
Pect. prof.	CF1	0.11		0.13	0.09
	SPE	0.09	0.12	0.19	0.12
Latiss. dors.	CF1	0.05		0.18	0.27
	SPE	0.26	0.07	0.17	0.46
Semispin. cap.	CF1	0.08		0.08	0.07
	SPE	0.16	0.10	0.10	0.09

(continued)

Table 7 (Continued)

Comparison of Intermediate and Mature Collagen Crosslink Quantitation in the Selected Muscles, Expressed as Moles Crosslink / Mole Collagen, following CF1 or PRS-SPE Cartridge Pre-fractionation and Quantitation by either Amino Acid Analysis of RPHPLC

		Mature X-Link		Intermediate X-Links	
Muscle	Pre-fract.	Pyr. (AAA)	Pyr. (RP, HPLC)	DHLNL	HLNL
Serr. vent. thor.	CF1	0.34		0.12	0.08
	SPE	0.16	0.30	0.16	0.06
Splenius	CF1	0.12		0.08	0.07
	SPE	0.23	0.15	0.11	0.06

PRS Cartridge Loading Capacity

Tables 6A and 6B show the influence of increased loading on the binding of hydroxyproline and the fluorescent crosslinks respectively.

As the load increases, so the proportion of hydroxyproline eluting in the loading buffer increases. Loading in excess of 3% of the sorbent dry weight results in approximately half the standard hydroxyproline not being retained on the cartridge in the pH 1.9 loading buffer. This was to be expected as hydroxyproline would be the least strongly adsorbed of the standard mixture. Provided that 3% of the sorbent dry weight or less, is loaded, then the first three eluants can be pooled and assayed to provide a value for percentage collagen in the parent tissue

The data in Table 6B shows the same trend as that in 6A, i.e. larger loadings result in earlier elution from the cartridges. However, it also shows that even if over 3% of the sorbent dry weight is loaded the fluorescent crosslinks do not converge upon the elution position of hydroxyproline.

It is interesting to note, that pyridinoline seems more susceptible to loading affects than pentosidine, presumably reflecting the latters stronger affinity for cation exchange sorbents.

CF1 Pre-fractionation v. PRS Solid Phase Extraction Cartridges.

Table 7 shows how closely, in the majority of cases, the two pre-fractionation methods agree when quantitating the intermediate collagen crosslinks by amino acid analysis and how far they differ when quantitating pyridinoline by the same analytical procedure. However, the quantitation of pyridinoline recovery from the PRS-SPE by RP HPLC, closely agrees with that from the CF1, indicating the presence of co-migrants in the PRS-SPE eluants that led to errors in quantitation by the amino acid analyser.

CONCLUSIONS

Of the 12 solid phase extraction sorbents tested, the propylsulphonic acid (PRS) cartridges proved most effective, being capable of resolving the basic crosslinking amino acids from the majority of acidic and neutral amino acids.

The development of a volatile elution buffer procedure, coupled with the extremely small elution volumes involved (3-5 mLs / eluant), permits rapid further processing after pre-fractionation. In fact, the elution buffer procedure can be further simplified, as the pH 3.1 and pH 6.5 eluants can be dispensed with, provided that sample loading is no more than 3% of the sorbent dry weight. In this case, pyridinoline and pentosidine are retained up to pH 4.7. Any residual material of potential interest can be desorbed from the cartridges using 4N ammonium hydroxide.

The recovery of standard pyridinoline and pentosidine was greater than that reported from either of the other pre-fractionation sorbents, CF1 and SP Sepharose.[7] In addition, the hydroxyproline recovery and resolution from the crosslinking components of a standard mixture have been shown to be consistent within an adequately wide range of sample loadings, for example, 3% of a 500mg cartridge represents a 15mg sample load. In our experience, such a loading would provide sufficient mature and intermediate crosslinks for analysis by the Alpha Plus amino acid analyser of tissues containing as little as 20% collagen. Analysis of pentosidine and pyridinoline by RP HPLC requires less than 300μg collagen, so 15mg of sample would provide sufficient fluorescent crosslinks from a tissue containing as little as 2% collagen. In addition the wide range of SPE cartridge sizes that are commercially available, up to 15g for example, allow the analysis of samples with even lower collagen contents.

The fact that pre-fractionation with PRS-SPE cartridges utilises the same chromatographic principle as one of the subsequent analytical tools, i.e. the amino acid analyser, provides a likely explanation for the poor agreement between the pre-fractionation procedures when assaying pyridinoline by the

Alpha Plus analyser. Presumably, components desorbed from PRS-SPE in the same eluant as the collagen crosslinks will co-elute with them on the amino acid analyser. CF1, on the other hand, exploits the differences in degree of hydrophobicity between the amino acids of interest and, therefore, subsequent amino acid analysis reveals fewer co-migrants. This suggestion is supported by the observation that the intermediate crosslinks, which elute later than pyridinoline from the Alpha Plus analyser, show much closer agreement between the pre-fractionation procedures because few of the PRS-SPE co-migrants are retained on the analytical column as long as they are. RP HPLC on the other hand, utilises a different chromatographic principle to the PRS ion-exchange cartridges and a different detection system to the Alpha Plus analyser for the analysis of pyridinoline, resulting in much closer agreement between the pre-fractionation procedures for pyridinoline quantitation.

The expected variation in intra-muscular collagen crosslinking with variation in muscle growth coefficient was not demonstrable, but the experiment did provide an opportunity to rigorously test the limitations of the PRS-SPE procedure as the collagen content of these whole muscles ranged between 0.8% and 2.2%. The PRS-SPE cartridges proved as reliable and capable of recovering the pyridinoline from the minute amount of collagen in these samples as the generally accepted CF1 pre-fractionation procedure. In addition, we have already demonstrated the superiority of the PRS-SPE cartridge for recovering standard pentosidine over that of the preferred alternative to CF1 for this type of analysis, i.e. SP Sepharose.

Propylsulphonic acid solid phase extraction cartridges are an inexpensive, rapid and reliable pre-fractionation sorbent which, when combined with the volatile organic buffer procedure reported here, provides higher recovery yields of both the mature collagen crosslink pyridinoline and the advanced glycation end-product pentosidine than previously reported. The very small volumes involved during processing and the sample loading capacity makes PRS-SPE a useful tool for subsequent quantitation of these fluorophores by RP HPLC.

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Received December 20, 1995
Accepted January 3, 1996
Manuscript 4050