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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMN SWITCHING METHOD FOR THE DETERMINATION OF HYDROXYPROLINE IN MEAT AND MEAT PRODUCTS

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SUMMARY

A method is described for the determination of 4-hydroxyproline in meat and meat products. The amino acid is converted to a sensitive fluorescent derivative with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. Chromatography of other (unwanted) amino acid derivatives is avoided by column switching, thereby shortening analysis time. The method is suitable for routine analysis and is applicable to the entire range of 4-hydroxyproline levels normally encountered in meat and meat products (0.05–12.5%).

INTRODUCTION

Estimation of the amino acid *trans*-4-hydroxy-L-proline (HYP) after protein hydrolysis is the accepted procedure for the estimation of collagen[1]. Until recently the most popular approach for the analysis of HYP was a colorimetric procedure involving oxidation of HYP to pyrrole followed by reaction with *p*-dimethylaminobenzaldehyde. There are however numerous problems associated with this approach as evidenced by the various modifications which use a range of oxidising agents[2–4], reagents for removal of excess oxidant, and extraction procedures[5].

More recently, derivatisation reagents coupled with high-performance liquid chromatography (HPLC)[6,7] have been introduced to overcome problems of sensitivity and specificity. In particular several workers[8,9] have made use of the fluorogenic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), which has the additional advantage of a much faster rate of reaction with secondary than with primary amines. Existing procedures have been confined to the analysis of collagen-rich[10] samples and/or entail a relatively long analysis time[11].

In this paper the NBD-Cl approach has been extended to the estimation of low levels of HYP, and column switching introduced to reduce chromatographic run time.

Column switching has been widely used in process gas chromatography for many years[12], and its first application to HPLC was published in 1973[13]. The principle of operation is to transfer a mobile phase fraction, via a switching valve from the outlet of one column to the inlet of another. This permits several different tech-

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niques to be carried out, the most popular of which include sample clean-up[14], trace enrichment[15], and multi-column chromatography[16,17] (column programming).

We have made use of column switching in the sample clean-up mode to provide a rapid, sensitive procedure applicable to a wide range of HYP levels in meat and meat products.

EXPERIMENTAL

Equipment

HPLC was performed with a Varian (Walton-on-Thames, U.K.) Model 5000 pump, fitted with a Rheodyne (Berkeley, CA, U.S.A.) Model 7120 valve and a Perkin-Elmer (Beaconsfield, U.K.) Model 3000 fluorescence spectrophotometer. The analytical column was a 15 cm × 0.49 cm I.D. reversed-phase column, packed with octadecyldimethylmonochlorosilane (ODS) bonded to 5-μm LiChrosorb Si-100.

For column switching, the above equipment was used in conjunction with a Milton Roy (Riviera Beach, FL, U.S.A.) mini-pump, a Rheodyne Model 7010 (switching) valve, and a 4 cm × 0.2 cm I.D. pre-column, packed with ODS bonded to 10-μm LiChrosorb Si-100. The stationary phase and columns were both prepared in the laboratory.

Reagents

All solvents and chemicals were of analytical reagent grade except for acetonitrile which was HPLC-grade (Rathburns, Walkerburn, U.K.). Ion-pair reagents were from Fisons (Loughborough, U.K.) or B.D.H. (Poole, U.K.). HYP was obtained from Sigma (Poole, U.K.) and NBD-chloride from Aldrich (Gillingham, U.K.). The mobile phase was acetate buffer–acetonitrile (85:15), containing 4.2 mmol ion-pair reagent per litre of mobile phase. The acetate buffer contained 53.5 ml 1 *M* hydrochloric acid and 50 ml 1 *M* sodium acetate, diluted to 250 ml with distilled water (pH 1.4).

Sample preparation and derivatisation

Samples of meat were minced to a paste and then converted to an acetone-dried powder[18]. The sample was then milled (using a 1-mm screen) to ensure homogeneity and hydrolysed by refluxing with 6 *M* hydrochloric acid for 22 h.

A 100-μl neutralised aliquot of the hydrolysate, containing *ca.* 0.5 μg HYP, was then derivatised at 60°C for 3 min using the procedure of Ahnoff *et al.*[10]. Typically, 20 μl of the derivatised sample was injected into the column.

Method validation

Experiments were set up to examine the reproducibility of derivatisation, derivative stability, and recovery of HYP from hydrolysates. Finally, data were compared, obtained from a wide range of samples by HPLC and by a colorimetric method, which until recently was the method of choice in this laboratory.

RESULTS AND DISCUSSION

Chromatography

Optimisation of the chromatographic conditions was carried out with chicken meat, containing 0.1% HYP before hydrolysis. At this low level, chromatographic interference may be considered to be at its maximum and the problems of resolution, therefore, at their most critical.

When a mobile phase of acetate buffer–acetonitrile (85:15) was used, the NBD derivative of HYP (NBD-HYP) was only partially separated from other, interfering compounds (Fig. 1). Resolution may be improved by a reduction in the level of acetonitrile, but the advantage gained is offset by a reduction in fluorescence response[10]. Resolution can be improved by using ion-pair reagents. The capacity ratio (k') value of the NBD-HYP decreases, whilst that of several interfering peaks increases when alkyl sulphonate is added to the mobile phase. Fig. 2 shows that a linear decrease in the k' of NBD-HYP is obtained by increasing the chain length of the ion pair. Optimum separation is achieved with octanesulphonate (Fig. 3). Conveniently, NBD-HYP is the first peak to be eluted under these conditions, but the problem is that analysis time is relatively long (34 min). In an effort to reduce analysis time, two approaches were tested: a gradient elution and column switching.

Gradient elution. By rapidly increasing the acetonitrile content of the mobile

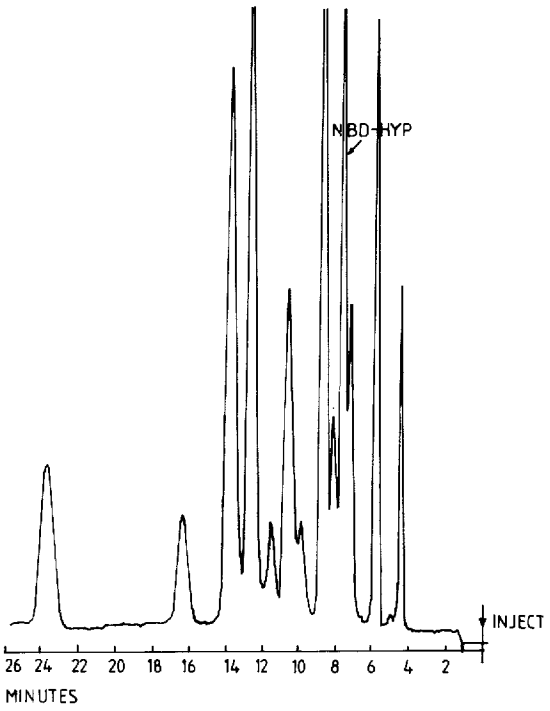

Fig. 1. Reversed-phase separation of NBD-HYP. Chromatographic conditions: column, 15 cm × 0.49 cm I.D. C_{18}; solvent, acetate buffer–acetonitrile (85:15); flow-rate, 1.5 ml/min; detection, fluorescence, Ex. 495 nm, slit 15 nm, EM 525 nm, slit 20 nm; injection volume, 20 μl; sample, chicken meat hydrolysate.

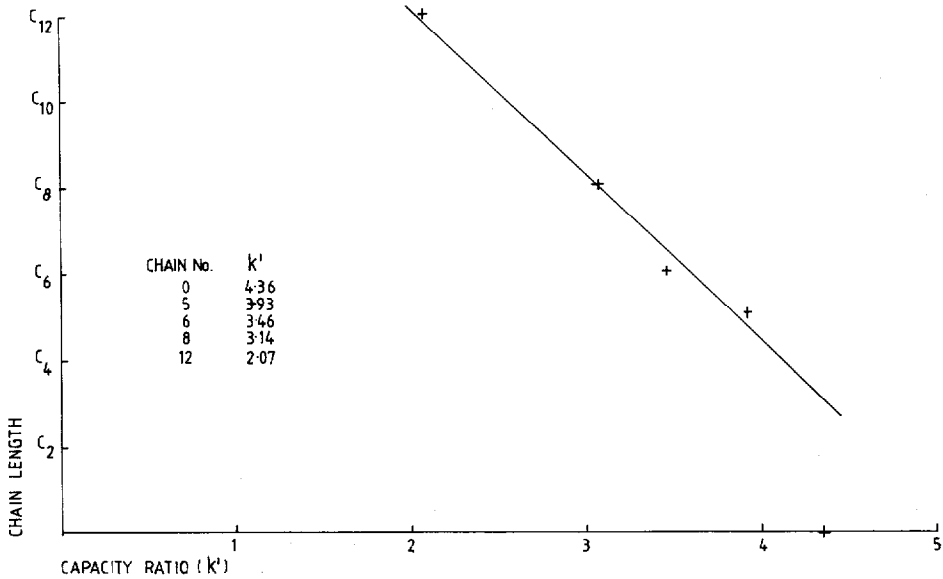

Fig. 2. Graph of the capacity ratio, k', of NBD-HYP *vs.* chain length of ion-pair reagent.

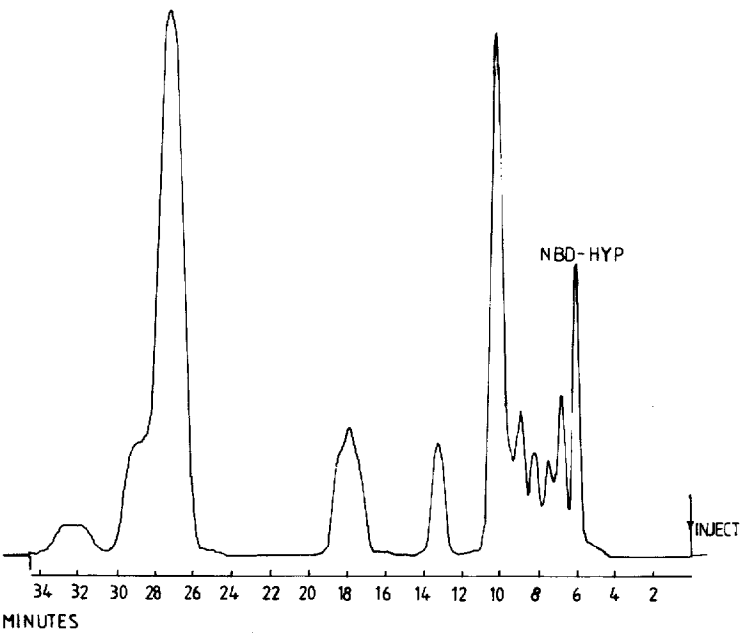

Fig. 3. Reversed-phase separation of NBD-HYP using sodium octane sulphonate ion-pair reagent. Chromatographic conditions: as for Fig. 1, except mobile phase contained 4.2 m*M* sodium octanesulphonate.

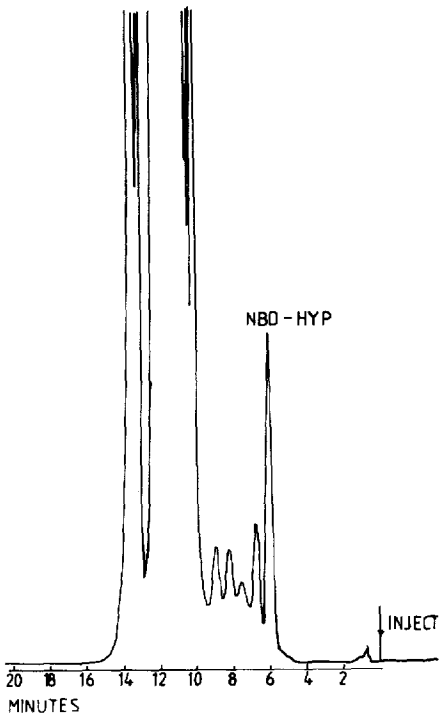

Fig. 4. Reversed-phase ion-pair separation of NBD-HYP with gradient elution. Chromatographic conditions: as for Fig. 3 except for use of a gradient: Acetonitrile content increased to 60% after 6 min, held for 5 min, and then returned to 15%.

phase following elution of NBD-HYP, the residual peaks can be rapidly eluted (Fig. 4). Allowing for re-equilibration, the analysis time in this approach is reduced from 34 to 20 min.

Column switching. A schematic diagram of the HPLC equipment used for column switching is shown in Fig. 5. Prior to injection, the injection valve is turned to the 'load' position and the switching valve to the 'inject' position, with pump A turned on and B off. With this configuration the pre-column and main analytical columns are connected in series, and the solvent flows via the dotted line through the switching valve. Following injection of the derivatised sample into the pre-column, NBD-HYP, the first peak to be eluted, is allowed to pass to the analytical column. The switching value is then turned to 'load' and pump B is turned on. Solvent from pump A travels directly to the analytical column (solid line through the switching valve), enabling NBD-HYP to be separated and detected in the normal manner. At the same time, the unwanted material that is still retained on the pre-column is eluted to waste by pump B. Due to the small dimensions of the column, this job is performed by the time NBD-HYP has been eluted from the analytical column, thus leaving the system ready for the next injection.

The net effect of this system is shown in Fig. 6. Only three residual peaks are eluted after NBD-HYP, and analysis time is reduced to *ca.* 10 min. In practice, the

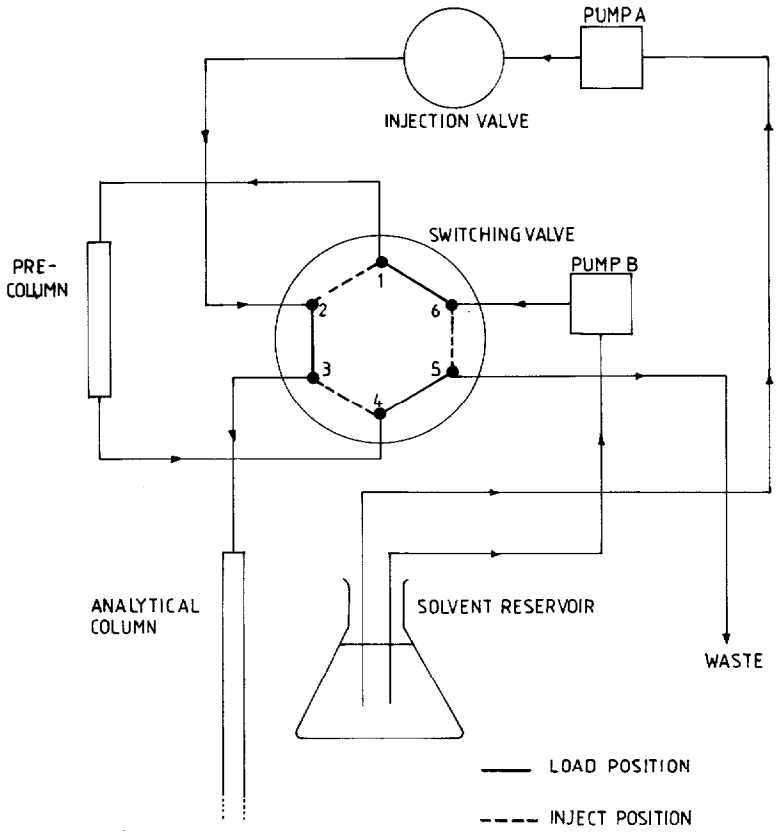

Fig. 5. Schematic diagram of column switching equipment.

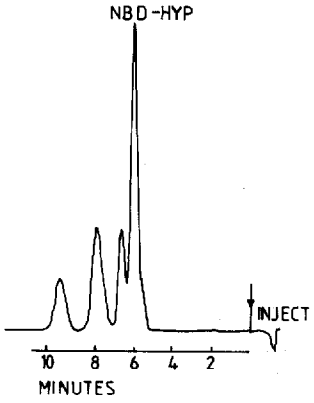

Fig. 6. Reversed-phase ion-pair separation of NBD-HYP with column switching. The equipment used is shown schematically in Fig. 5. Chromatographic conditions: As for Fig. 3, with the following additions; pre-column, 4 cm × 0.2 cm I.D., 10-μm C_{18}; Rheodyne switching valve; and Milton Roy mini-pump. Switching valve turned 40 s after injection and mini-pump adjusted to a flow-rate of 1.5 ml/min.

TABLE I

RECOVERY OF HYP FROM HYDROLYSATES

	Level of addition (μg)	*Level found (μg)*	*Recovery (%)*	*Mean*
Chicken hydrolysate(0.05% HYP)		0.10		
	0.1	0.19, 0.19	90, 90	90
	0.25	0.36, 0.35	104, 100	102
	0.50	0.58, 0.61	96, 102	99
Pork hydrolysate (0.16% HYP)		0.06		
	0.1	0.16, 0.16	100, 100	100
	0.25	0.315, 0.315	102, 102	102
	0.50	0.59, 0.53	106, 94	100

time between sample injection and column switching is 20–30% greater than that required for NBD-HYP to be eluted from the pre-column. This provides a margin for error and explains the accompanying residual peaks.

Other workers[11] have used UV detection at 495 nm rather than fluorescence. We found this to be *ca.* five times less sensitive, and whilst convenient for the analysis of higher HYP levels, it would have required the inclusion of a concentration step to detect the lower ones.

Using fluorescence detection, it was possible to detect as little as 1.5 ng (12 pmole) HYP in chicken breast hydrolysate. However, it should be stressed that background interference rather than the absolute detection level is the limiting factor in the analysis, and this varies according to the sample.

Derivatisation

The NBD-HYP peak height was stable over a period of 30 min when standards were stored in ice and shielded from light. After 60 min, a small (*ca.* 5%) loss was noticed. For routine analysis, samples were injected within 5–10 min of derivatisation. The derivatisation itself is very reproducible. The coefficient of variation for a series of 1.0-μg/ml HYP standards was only 2.0% with column switching and 2.9% with gradient elution.

Recovery of hydroxyproline

The recovery of HYP from protein hydrolysates is shown in Table I. Recovery experiments were carried out using two different protein hydrolysates, with measured additions of HYP at levels equal to, 2.5 times, and 5 times the indigenous level. Good recoveries were obtained for all three levels of addition to the pork hydrolysate, which had an indigenous level of 0.16% HYP. The lowest level of addition to chicken hydrolysate gave a rather low recovery of 90%, but the indigenous HYP level (0.05%) represents the lowest encountered during routine analysis.

Comparison with the colorimetric method

Table II shows a comparison of results obtained by HPLC with column switching and by the colorimetric method of Stegemann and Stalder[19]. The samples ranged from pure muscle (chicken breast, 0.05% HYP) to beef tendon (gristle No. 10, 12.3%

TABLE II

COMPARISON OF DATA BY THE STEGEMANN AND STALDER[19] AND HPLC PROCEDURES

Sample	*Hydroxyproline level (%)*	
	HPLC	*Stegemann and Stalder*[19]
Chicken breast (1)	0.04	0.05
Chicken breast (2)	0.04	0.05
Chicken breast (3)	0.07	0.09
Chicken breast (4)	0.07	0.06
Turkey breast	0.06	0.04
Mechanically recovered turkey meat	0.06	0.08
Turkey liver	0.04	0.06
Chicken leg (1)	0.10	0.11
Chicken leg (2)	0.14	0.19
Chicken leg (3)	0.19	0.19
Turkey heart	0.11	0.12
Turkey thigh	0.17	0.17
Turkey light trim	0.18	0.19
Turkey skin	0.67	0.73
Beef gristle (1)	2.96	2.66
Beef gristle (2)	3.92	4.22
Beef gristle (3)	4.37	4.60
Beef gristle (4)	4.14	4.52
Beef gristle (5)	5.22	4.88
Beef gristle (6)	6.04	6.66
Beef gristle (7)	11.24	10.32
Beef gristle (8)	10.71	10.83
Beef gristle (9)	12.05	11.88
Beef gristle (10)	12.34	11.46

HYP). Good agreement was obtained at all levels. Application of the *t*-test showed that there was no statistical difference between the two sets at the 95% confidence limit.

CONCLUSIONS

The major criteria for the estimation of HYP in protein hydrolysates are those of high sensitivity and specificity, combined preferably in the form of a rapid analysis. In these respects, derivatisation with NBD-Cl, followed by HPLC with column switching is a very convenient approach. NBD-Cl reacts rapidly to provide a stable fluorescent derivative that allows detection of picomole quantities of HYP. More importantly, since the rate of reaction with secondary amines is an order of magnitude faster than with primary amines, there is a concomitant reduction in the level of interference from primary amines during chromatography. A lengthy chromatographic run can be conveniently avoided by the use of column switching. There is

also a hidden benefit in that the pre-column removes impurities (from derivatisation and solvent) and thereby considerably prolongs the lifetime of the analytical column.

The technique has now been in routine use in our laboratory for eighteen months and has presented few problems. It is applicable to a very wide range of HYP levels in meat and connective tissue, and since it requires only microlitre quantities for derivatisation, it is equally applicable to the analysis of very small (milligram) samples of protein.

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