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Technical Note

A Method for the Reverse Phase High Performance Liquid Chromatography of Peptides from Cheddar Cheese

ABSTRACT

A water-soluble nitrogen fraction (WSNF) was isolated from Cheddar cheese by a combination of extraction with water, methanol precipitation, removal of lipid with hexane, and permeation chromatography. The resultant product was fractionated using reverse phase fast protein liquid chromatography (FPLC). Processing of replicate cheese samples yielded final reverse phase chromatograms that were reproducible. Chemical analysis of an extract fractionated by reverse phase chromatography established that most peaks detected were either polypeptides or amino acids. The developed procedure may be suitable for carrying out time course profile studies of peptides produced during the accelerated ripening of Cheddar cheese.

INTRODUCTION

Cheese flavour research concerning the non-volatile fraction of Cheddar cheese is handicapped by lack of relatively easy procedures for the final fractionation of the water-soluble nitrogen fraction (WSNF). Extensive systematic evaluation of various methods for the isolation and analysis of the WSNF-containing proteins, peptides and amino acids has been reported in a series of papers by Kuchroo & Fox (1982*a*, *b*, 1983*a*, *b*). It is apparent from this work that the evaluation of WSNF by a single analytical procedure remains impossible.

Champion & Stanley (1982) used gel permeation and preparative reverse phase HPLC to isolate and detect bitter peptide fractions; however, the final

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HPLC took about 80 min per sample. Pham & Nakai (1984) correlated reverse phase HPLC fractionation profiles with cheeses of four ages but in this case the sample processing was rather time-consuming.

In the present work we have attempted to develop sample work-up and HPLC chromatographic conditions that can be used to screen the large number of samples that may be encountered during investigation into the accelerated ripening of Cheddar cheese (Law, 1984).

The sample work-up procedure adopted is a modification of that developed by Kuchroo & Fox (1982*a*) for the isolation of the WSNF of cheese. Water extraction followed by fractionation of a methanol-soluble phase by gel permeation chromatography was used. Methanol was substituted for ethanol because the former can readily be obtained at a purity satisfactory for reverse phase FPLC and is more volatile than ethanol. Concentration of large volumes of relatively impure solvents used during the precipitation stage could lead to artifact peaks eluting from the reverse phase column. Sephadex G25 was used because Kuchroo & Fox (1983*a*) found that it gave more peaks than Sephadex G50, G150 or Sepharose 6B fractionation. Sephadex G10 was avoided in order to minimise adsorption of peptides. FPLC was substituted for DEAE chromatography and SDS gel electrophoresis because of its superior resolution powers, possibility of automation and the potential as a single analytical method.

MATERIALS AND METHODS

Cheddar cheese was made with a single strain starter (*Streptococcus cremoris*, NCDO 1198) using standard commercial rennet extract (Chapman & Burnett, 1972). The cheese was waxed and stored at 12°C and 85–87% relative humidity for twelve months, then at −20°C prior to analysis. A second cheese was prepared using the accelerated ripening system developed by Law & Wigmore (1983) and marketed by Imperial Biotechnology Ltd., South Kensington, London (Accelase). It was allowed to ripen 8 months at 12°C then a further 3 weeks at room temperature.

HPLC grade water, methanol and hexane were obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Cheese (80 ± 0·1 g) with a moisture content of 37% was blended for 12 min with HPLC grade water (100 ml) in a 350 ml stainless steel beaker (Phillip, Harris Scientific, Park Royal, London, England) surrounded by ice, using a model L2R Silverson homogeniser (Silverson Machines Ltd, Waterside, Chesham, Bucks, England) equipped with a 5/8 in diameter head. The product was alternately homogenised (2 min) and cooled (2 min) in order to minimise a tendency for the temperature to rise. After centrifugration at 2000 *g* (max) for 15 min at 4°C

the supernatant below the crust of fat was removed using a 20 ml glass syringe fitted with a metal needle. The supernatant was strained through glass wool then filtered at approximately 3300 Pa (25 mm Hg) using a 7·0 cm No. 4 Whatman filter circle (Whatman Ltd, Maidstone, Kent, England) all at 4°C. Iced HPLC grade methanol (92 ml) was added dropwise to the supernatant (40 ml) cooled by ice, with rapid overhead stirring during a period of 10 min. After a further 35 min of slow stirring, the product was centrifuged at 25 000 *g* (max) for 30 min at 4°C. The total precipitation time was not necessarily optimal and was arbitrarily set at 45 min. The supernatant was rotary-evaporated at an initial temperature of 10°C rising to 40°C at approximately 130 Pa (1 mm Hg). Freeze-drying was considered unnecessary because the rennet and other enzymes would have been denatured during the methanol precipitation step.

Lipid was removed from the film of crude peptide extract deposited on the walls of the evaporator flask by extracting twice with HPLC grade hexane (50 ml) for 5 min, discarding the liquid phase and removing residual organic solvent on a rotary evaporator. The residue was extracted with H_2O (3·5 ml), swirling with 5 mm diameter glass balls (BDH Ltd, Poole, Dorset, England) to dislodge the residue, for 10 min at 4°C, then passed through a 25 mm diameter 3 μm MF-Millipore filter (Millipore Ltd, Harrow, Middlesex, England) and made to 4·5 ml with water. It was then deep-frozen and immediately before the next stage was thawed and any precipitate was removed by centrifugation at 2500 *g* (max) for 5 min at room temperature followed by membrane filtration as before if necessary.

Further reduction in the concentration of protein was achieved by permeation through a 30 × 1·6 cm bed of fine G25 Sephadex (Pharmacia Ltd, Midsummer Boulevard, Milton Keynes, England) eluted with HPLC grade water. Calibration was carried out with bovine serum albumin (6 mg ml^{-1}) in aqueous 0·2M acetone. A 2·0 ml sample of calibration mixture or crude peptide extract was passed through the column at 8 ml h^{-1} and 2·0 ml fractions were collected. The elution volumes of bovine serum albumin and acetone were designated V_0 and V_i respectively. Band I, which should be rich in protein and protein-sized molecules, consisted of the first fractions pooled to a volume of 1·25 V_0 ml. Band II was assumed to consist mainly of peptides, amino acids and salts with a molecular size less than *ca.* 8000 Daltons and contained pooled fractions eluting between 1·25 V_0 ml and 3 V_i ml. Both bands were concentrated by rotary evaporation from a water bath set at 40°C at 130 Pa (1 mm Hg), then made to 10 ml. The composition of band I was not investigated further.

Lipid extraction tests were carried out on four separate samples taken from the cheese prepared by the conventional cheesemaking method without any accelerating enzyme treatment. Two peptide extracts were

treated twice with hexane only (the standard procedure). Two more were initially treated twice with diethyl ether in order to check that the hexane was completely removing the lipid. This was followed by reconstitution of the film with water (10 ml) to release lipid mechanically trapped by the film, re-evaporation and re-extraction twice with hexane.

Analytical reverse phase chromatography of cheese extracts

The Pharmacia Fast Protein Liquid Chromatograph (FPLC) was used for the final analysis. It consisted of a GP-250 gradient programmer, two P-500 high precision pumps, a 4 ml min^{-1} mixing chamber, a V-7 loading valve, a UV-1 single path monitor fitted with a 10 mm path length flow cell and a REC-482 two channel recorder (Pharmacia Ltd, Midsummer Boulevard, Milton Keynes, England). This equipment was fitted with a Pharmacia Pep RPC HR 5/5 reverse phase column 5 cm long and 5 mm diameter. The packing consisted of silica with a pore size of 100 Å and a C_2/C_{18} coating. The solvent used for elution was passed through the reference cell before entering the column in order to partially compensate for baseline drift, even though this may expose the reference cell to excessive pressure. The absorbance range was always set at $A_{214} = 2{\cdot}00$ in order to produce the straightest possible baseline.

The eluate system consisted of solvent A, water with or without 0·1% v/v trifluoroacetic acid (TFA) (Spectrosol grade, BDH Chemicals Ltd, Poole, England) and solvent B, methanol with or without 0·1% v/v TFA. A flow rate of 0·7 ml min^{-1} at pressures rising to approximately 3·5 MPa was used. The concentration of solvent B in the eluent used for the analytical gradient, shown in brackets after the accumulated volume of eluent, was as follows: 0 ml (0%), 4 ml (0%), then 15 ml (30%). This was followed by 30 ml (100%), 50 ml (100%), 52 ml (0%) and the flow was stopped at 62 ml (0%). This was designed to remove the last traces of the sample from the column and re-equilibrate with the initial aqueous solvent. Suitably diluted samples in starting solvent were passed through a 0·22 μm Millipore Millex-GV disposable filter directly into the 50 μl loading loop and then onto the column. Usually about 1–2 mg of crude peptide was loaded onto the column for each analytical separation.

Preparative reverse phase chromatography of cheese extract

In order to confirm that the processing procedure produced samples that consist mainly of peptides and free amino acids, a sample of overripened Cheddar cheese was processed in the previously described manner. A very shallow gradient was used with a flow rate of 0·7 ml min^{-1}. Defining the

gradient using the previously used format, the parameters were 0 ml (0%), 31 ml (10%), 32 ml (100%), 52 ml (100%), 54 ml (0%), then 64 ml (0%). A suitably diluted sample (50 μl) was repeatedly chromatographed and approximately 0·25 ml fractions were automatically pooled in the same tubes in order to build up sufficient material to allow for chemical analysis.

Analysis of reverse phase fractions with ninhydrin reagent

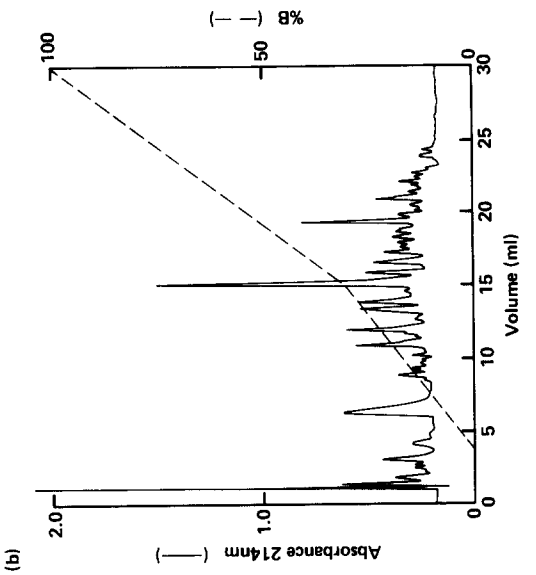
Ninhydrin reagent was prepared according to the method of Moore & Stein (1954) and stored under nitrogen at −20°C in a brown bottle whose screw cap was lined with a silicone rubber seal to exclude air. The assay was carried out as follows. To eluate (0·1 ml) and pH 5·52 4M sodium acetate trihydrate (0·1 ml) in ice was added ninhydrin reagent (0·1 ml) followed by heating in a boiling water bath for 10 min. The reaction mixture was allowed to cool a little, diluted with 50% aqueous ethanol (1 ml), shaken for 30 s to oxidise any excess hydrindantin and the A_{570} was measured using 50% aqueous ethanol as reference. Fractions earlier than tube 45 were not assayed because of unavailability of material.

Analysis of reverse phase fractions with alkaline hydrolysis followed by ninhydrin

A modification of the procedure of Fruchter & Crestfield (1965) was used. Column eluate (0·1 ml) and 0·1M NaOH (1·0 ml) in an uncovered polypropylene centrifuge tube was heated at 110 ± 2°C overnight. The residue and 30% v/v aqueous acetic acid (0·1 ml) was placed in a boiling water bath for 6 min. To this extract was added pH 5·52 4M sodium acetate trihydrate buffer (0·1 ml) and ninhydrin solution (0·1 ml) which was then treated in the same way as in the previous section.

Analysis of reverse phase fractions using Lowry reagent

Column fractions, after concentration, were assayed using the method of Lowry *et al.* (1951). Samples of column fractions (0·4 ml) were dried in an oven at 110°C for 30 min and reconstituted with water (0·2 ml). To this was added the reagent C (1·0 ml) of Lowry *et al.* (1951) and the mixture stored at room temperature for 10 min. Reagent E (0·1 ml) was added directly into the vortex during continuous mixing and the A_{750} against water was read after 30 min at room temperature.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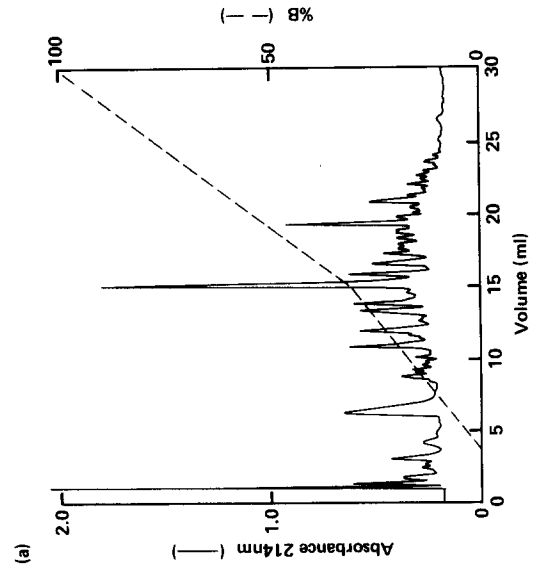
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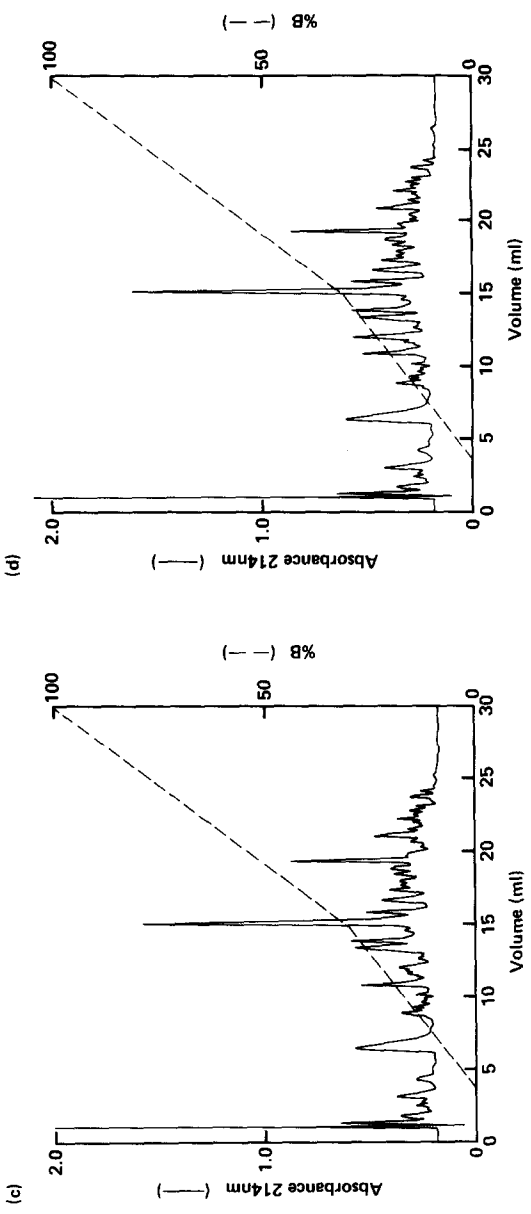

Fig. 1. Reverse phase separation of peptides extracted from four separate samples of conventionally made Cheddar cheese. Peptide film extracted twice with hexane ((a) and (b)). Peptide film extracted twice each with diethyl ether followed by hexane ((c) and (d)). The gradient was formed from 0·1% v/v TFA in water and 0·1% v/v TFA in methanol. The line indicating the gradient (– – –) gives the percentage content of 0·1% v/v TFA in methanol (solvent B). Absorbance of effluent at 214 nm (——).

RESULTS AND DISCUSSION

The reproducibility of the sample work-up procedure was excellent as demonstrated from replicate analysis by reverse phase chromatography of two samples of cheese after extraction with hexane only (Fig. 1(a) and (b)) and two more cheese samples where extraction was with diethyl ether followed by hexane (Fig. 1(c) and (d)). This latter additional extraction with diethyl ether produced no evidence of further removal of lipid.

Components that responded positively to Lowry reagent and ninhydrin reagent with and without alkaline hydrolysis were detected (Fig. 2). Some components gave a higher response with ninhydrin *after* compared with *before* alkaline hydrolysis which is one characteristic of peptides (Fig. 2). Furthermore, alkaline hydrolysis followed by ninhydrin gave peaks at least twice as high as with Lowry reagent and in some instances revealed peaks not detected by the latter reagent. Alkaline hydrolysis followed by ninhydrin also required less sample than the Lowry assay (0·1 ml compared with 0·4 ml).

Acid hydrolysates (6M HCl with heating for 18 h at 110°C) were initially evaluated for amino acid content by thin-layer chromatography on cellulose (Levy & Chung, 1953) with butanol–acetic acid–water (4:1:5) and finally quantitated by automatic ion-exchange analysis (Table 1). Most peaks detected by monitoring the eluate from the reverse phase column at 214 nm (Fig. 2(a)) and by reaction with ninhydrin after alkaline hydrolysis (Fig. 2(b)) contained amino acids before and/or after acid hydrolysis (Table 1). The early fractions tended to be rich in free amino acids. Thus fractions 5 and 6 contained free aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, histidine, lysine and arginine with very little evidence of amino acids released by acid hydrolysis. Individual free amino acids predominated in some fractions, valine in fraction 7, methionine in fraction 8, isoleucine in fractions 15 and 16, leucine in fractions 15, 16 and 17. Free phenylalanine was found in fractions 33, 34 and 35 and was detected directly at 214 nm, giving the largest peak shown in Fig. 2(a). From about fraction 40 onwards substances from which amino acids were released by both acid and alkaline hydrolysis tended to predominate although the concentrations were low compared with earlier fractions.

Some aspects of this analytical procedure could be improved. One important requirement of future work is to be able to taste the individual fractions after removal of solvents in order to detect flavour peptides. The greatest number of peaks observed with reverse phase chromatography was obtained with the ion pairing agent trifluoroacetic acid which, if used, would interfere with organoleptic testing. One solvent system avoiding this problem is methanol and water without ion pairing agents; however,

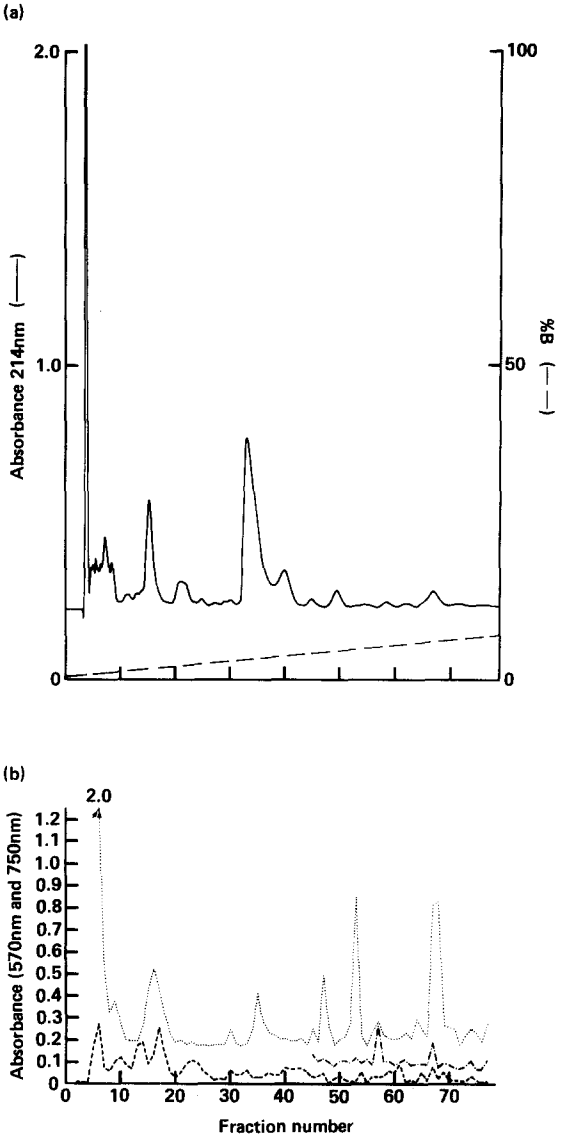

Fig. 2. Reverse phase separation of peptides extracted from an overripened Cheddar cheese accelerated by addition of enzymes. (a) The gradient was formed from 0·1% v/v TFA in water and 0·1% v/v TFA in methanol. The line indicating the gradient (– – –) gives the percentage content of 0·1% v/v TFA in methanol (solvent B). Absorbance of effluent at 214 nm (——). (b) Chemical analysis of reverse phase fractions (0·25 ml approx) using Lowry reagent (- - - - -), ninhydrin (– · – · –), and alkaline hydrolysis followed by ninhydrin (· · · · · ·).

TABLE 1

Free and Total Amino Acids in Reverse Phase Fractions (nmole ml^{-1})

(Blank spaces represent amino acids not detected or concentrations below 1 nmol ml^{-1}. Correction has been made for buffer blank values)

Amino acids	*Mean blank*	*5*		*6*		*7*		*8*		*9*		*10*		*15*	
		Free	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*
Asp	0·20	49·8	68·9	14·9	38·2		14·8		5·18				10·4		9·42
Thr	0·19	26·8	9·60	10·5	9·44		7·44		6·54				4·12		3·82
Ser	0·31	12·8	11·1	4·88	9·20		12·8		4·18				4·42	1·46	5·52
Glu	0·14	115·0	158·0	52·5	134·0	1·88	38·1		77·5				21·7		18·4
Gly	0·42	21·0	26·6	4·30	19·1		17·1		4·72				3·58		8·54
Ala	1·12	257·0	227·0	109·9	116·0	5·96	13·4	1·38	2·72	1·92	2·90		3·90		2·30
Val	1·32			20·4	22·5	262·0	243·0	77·8	81·7	4·92	7·70		6·22		5·32
Met	0·12			4·64		5·66	5·64	88·8	63·0	27·5	21·4	2·40	3·06	1·84	2·14
Ileu	0·26						6·70		1·08		4·20	27·5	32·6	177·0	171·0
Leu	0·36						13·0				7·24	5·46	11·6	259·0	247·0
Tyr	4·82										7·54		4·74		
Phe	1·42						7·04							1·34	17·7
His	4·06	37·2	29·6	118·0	129·0	51·3	61·7		4·08		1·44		0·36	1·76	
Lys	0·35	43·5	73·9	12·4	47·2	2·98	12·2		4·94		3·98		15·8		15·7
Arg	0·38	5·90	4·52	2·48	5·20		7·30		1·79		2·42		3·02		2·06

continued

TABLE 1—*contd.*

Amino acids	*16*		*17*		*21*		*33*		*34*		*35*		*39*		*40*	
	Free	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*
Asp		2·88		2·78		2·20		5·18		3·90		3·32		8·44		1·58
Thr		2·44		2·18		1·26		2·62		2·12		1·62		2·38		2·10
Ser		2·24		2·14		1·72		3·24		3·30		4·36		4·76		1·82
Glu		9·00		7·38		6·32		10·1	1·32	7·16		5·96		12·3		2·70
Gly		2·76		2·64		3·56		5·66		4·90		7·20	1·76	20·3		3·96
Ala								1·40				2·44		6·24		
Val		2·84				4·68		5·00		3·56				5·38		1·66
Met						1·66		3·02		1·28						
Ileu	53·6	53·4	8·32	10·1		1·90		2·84		2·86		3·06		4·28		
Leu	261·0	297·0	84·9	97·0	1·10	3·18		3·18		2·48		6·68	1·38	9·76		3·36
Tyr					1·18								7·04			
Phe					1·58		21·1	27·6	102·0	92·3	83·3	76·7		3·70		1·58
His									1·60	1·02						
Lys		2·92				1·86		5·46		2·94		1·64				
Arg				2·22												

continued

TABLE 1—*contd.*

Amino acids	*44*		*47*		*50*		*53*		*58*		*63*		*67*		*68*	
	Free	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*	*Free*	*Total*
Asp		7·30		7·44		3·89		2·66		2·58		3·46				2·26
Thr		5·98		4·78		2·16		1·44		2·00						
Ser		11·2	1·28	16·6		4·74		3·48		2·22		4·06				2·16
Glu		13·1	1·56	8·18		9·90		4·02		4·12		4·90		2·14		4·92
Gly		13·0		14·3		7·98		5·98		3·34		6·94		5·18		8·92
Ala		4·80		4·04		1·18		1·96				2·28		1·30		1·64
Val	1·10	8·36		5·94		5·56		4·30				3·68		4·92		5·80
Met		2·34		3·48		1·64		1·98				1·86				
Ileu		3·26		2·98		2·28		1·20		3·24						
Leu		7·66		5·62		3·12		2·66				2·48	1·82	2·48	1·74	2·18
Tyr			1·18							4·74			1·14		1·30	
Phe		10·3		8·26	1·10	10·0		7·44				10·24				
His		2·56	1·30	1·86												
Lys		5·84		4·98										1·50		2·78
Arg		5·74		1·08												

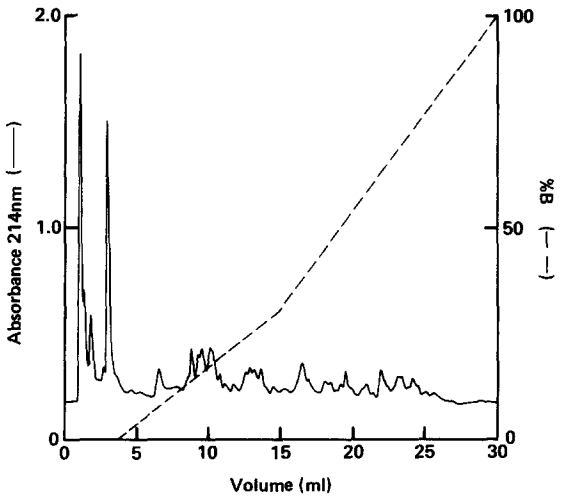

Fig. 3. Reverse phase separation of peptides extracted from conventionally made Cheddar cheese. The gradient was formed from water and methanol. The line indicating the gradient (– – –) gives the percentage content of methanol (solvent **B**). Absorbance of effluent at 214 nm (——).

peptides chromatographed using this eluent exhibited very short elution volumes (Fig. 3), and the resultant chromatogram was relatively featureless. The reason for this may be because the column is too short (5 cm) because Champion & Stanley (1982), using a 30 cm column eluted with methanol and water, successfully fractioned peptide mixtures obtained from Cheddar cheese into many separate components. Future work should use methods which overcome some of these problems.

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