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Evaluation of solid-phase extraction procedures in peptide analysis

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Abstract

Solid-phase extraction (SPE) procedures for peptide isolation and fractionation, based on non-polar and ionic interactions, were evaluated using small synthetic peptides and casein enzymatic hydrolysates. SPE based on hydrophobic phases is a useful, efficient and rapid procedure for peptide extraction and concentration. It allows a successful peptide fractionation using eluents that contain an increasing content of acetonitrile in the presence of trifluoroacetic acid. Differences regarding selectivity are observed between sorbents. Non-polar interaction with C_{18} sorbents is adequate for the isolation of very polar and hydrophobic peptides. CN sorbents are only adequate for very hydrophobic peptides. PH, CH, C_8 and C_2 sorbents are useful for isolating and fractionating hydrophobic and very non-polar peptides, but generally not for very polar peptides. Ionic solid-phase extraction using Accell Plus cartridges of QMA (quaternary methylammonium) and CM (carboxymethyl) are very useful for the fractionation of peptide mixtures into basic, acidic and neutral pools of peptides. It can be concluded that SPE using these procedures is a useful tool for the isolation and fractionation of peptides from biological and food samples.

1. Introduction

Sample preparation columns based on the principles of solid-phase extraction (SPE) are fast becoming indispensable tools in many areas of research [1]. This technique provides a clean and concentrated extract that simplifies subsequent analysis by removing interfering or cross-reacting components. The use of SPE is growing rapidly because of the current availability of many bonded silica sorbents [2]. These materials make SPE an extremely selective and efficient technique when used before radioimmunoassays, HPLC, GC, MS, TLC or bioassay.

SPE has proved very useful for isolating peptides from biological tissues [3,4]. It appears also to be very promising technique for the extraction and preferential enrichment of peptides with an application to food samples [5–9]. Bennett [10] used octadecylsilica (ODS) cartridges followed by a sequence of a variety of RP-HPLC solvent systems for exploiting the hydrophobic, basic and acidic character of the peptides to be purified. ODS SPE columns have become an important tool in removing salt and polar materials following tissue homogenization, while preferentially enriching the oligopeptide fraction [11]. Higa and Desiderio [12] optimized the recovery of substance P and methionine-enkephalin synthetic peptides from an ODS dispos-

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able cartridge. Optimization of SPE with C_{18} columns has also proved useful for the extraction of peptides from dairy samples [5]. Herraiz et al. [7] recently separated peptides by RP-HPLC following peptide isolation from skim milk using Sep-Pak C_{18} cartridges. Prefractionation of complex mixtures of peptides from cheese using SPE on ODS after gel filtration chromatography has also been reported as a useful technique for the further identification of peptides [8].

In addition to non-polar SPE cartridges, other silica-based ion-exchange Sep-Pak cartridges packed with either a carboxymethyl (CM) cation exchanger or a quaternary methylammonium (QMA) anion exchanger have already been successfully used for the fractionation of pituitary peptides into basic, neutral and acidic pools [13].

An adequate purification of biological samples with a low content of biologically active peptides requires the use of crude fractionation procedures preceding high-resolution RP-HPLC. In addition to the concentration effect obtained with the extraction process, the benefit of the prefractionation of peptides for selective chromatographic resolution has been reported [6]. Many types of bonded materials are currently available that may lead to different selectivities when applied to peptide extraction. Thus, owing to the increasing possibilities of SPE, new research is currently needed to investigate the behaviour of different available SPE sorbents in the isolation and fractionation of peptides. A systematic approach to the fractionation of peptides using SPE would be very useful, because of the heterogeneity of peptides in complex mixtures as biological and food samples. For obtaining a high recovery of a purified peptide, it is necessary to identify the most suitable bonded silica phase and elution conditions. In this regard, the possibility of non-specific irreversible adsorption altering the extraction should be considered [5].

The purpose of this work was to study the isolation and fractionation of synthetic peptides and peptides from casein enzymatic hydrolysates by using SPE with various types of bonded silica phases. This study may contribute to the development of systematic methods for the isolation and prefractionation of peptides in complex mixtures.

2. Experimental

2.1. Synthetic peptides and casein enzymatic hydrolysates

Glutathione, V-G-S-E, G-G-Y-R, K-W-K, P-F-G-K, V-H-L-T-P-V-E-K, (L,D)-L-G-(L,D)-F, W-W, D-R-V-Y-I-H-P-F-H-L-L-V-Y-S, F-L-E-E-V, F-L-E-E-I and F-L-E-E-L were obtained from Sigma (St. Louis MO, USA) and G-G-W-A from Serva (Heidelberg, Germany). Peptides were used without further purification and were selected to cover a range of different size, hydrophobicity (peptides with polar and non-polar amino acids), charge at the pH used (peptide with acidic and basic amino acids) and retention times in a C_{18} RP-HPLC column.

Complex mixtures of peptides were obtained from enzymatic hydrolysates from commercial α- and β-casein as follows: a 1.66 mg/ml concentration in 10 m*M* phosphate buffer (pH 8) of each casein (Sigma), used without further purification, was mixed with 0.5 mg of trypsin from porcine pancreas (Type II crude, Sigma) dissolved in the same buffer (approximate enzyme-to-substrate molar ratio = 1:110). Trypsin was not treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) and has a significant residual activity of chymotrypsin. Proteolysis was carried out at 37°C in a water-bath for 5 h. The enzymatic hydrolysates obtained (30 ml) were acidified to pH 2 with trifluoroacetic acid (TFA) and stored at −20°C until further utilized for SPE. Then, portions (2 ml) were passed through the SPE columns at 1 ml/min. Solutions used as reaction controls were prepared independently with α- and β-casein, but without the addition of trypsin. In the same manner, controls were prepared but lacking the casein.

2.2. SPE disposable cartridges and columns

Disposable SPE columns containing 500 mg of sorbent (40 μm, 60 Å) with a column volume of

2.8 ml were obtained from Varian (Harbor City, CA, USA), containing the following sorbents: C_2(ethyl), C_8(octyl), C_{18}(octadecyl, 18% carbon loading), PH (phenyl), CH (cyclohexyl), CN (cyanopropyl, non-end-capped silica) and CBA (carboxylic acid). Sep-Pak Plus environmental C_{18} cartridges (820 mg of sorbent, 80 μm, 125 Å; 12% carbon loading) and Accell Plus QMA (quaternary methylammonium) and Accell Plus CM (carboxymethyl) ion-exchange Sep-Pak cartridges (360 mg of sorbent, 45 μm, 300 Å) were obtained from Waters (Milford, MA, USA).

CN, C_2, PH, CH, C_8, C_{18}, Sep-Pak Plus C_{18} columns and cartridges were conditioned prior to peptide loading by passing successively 2 ml of methanol (Scharlau, Barcelona, Spain), 2 ml of Milli-Q-purified water and 2 ml of a solution of 0.1% of protein sequencing grade TFA (Sigma) in water. The void volumes were ca. 0.6 ml for Varian SPE columns, 0.8 ml for CM and QMA cartridges and 1.6 ml for Sep-Pak Plus C_{18} cartridges. CBA columns were conditioned with 2 ml of methanol and subsequently with 2 ml of 10 m*M* ammonium acetate buffer (pH 5.5). CM and QMA cartridges were conditioned by passing 2 ml of Milli-Q-purified water and subsequently 2 ml of 10 m*M* ammonium acetate buffer (pH 5.5).

2.3. SPE procedures of synthetic peptides

SPE of synthetic peptides was studied using the following procedures:

(a) A reference sample was obtained by dissolving synthetic peptides in 0.1% of protein sequencing grade TFA (Sigma) in Milli-Q-purified water to provide a peptide concentration of 0.01–0.1 mg/ml. Portions of 2 ml of this solution were loaded and passed slowly (1 ml/min) in duplicate through the following disposable cartridges: C_2, C_8, C_{18}, CN, PH, CH and Sep-Pak environmental C_{18}. The peptide concentration used is adequate for further direct HPLC analysis, allowing an accurate measurement of peptide recovery after SPE. The amount applied did not overload the SPE columns. The unretained fraction was recovered from the outlet of the SPE columns. Then, retained peptides on the sorbent were washed with 2 ml of 0.1% TFA in water. Subsequently, peptides were eluted with fractions containing a stepwise gradient in acetonitrile (ACN) (Scharlau, Barcelona, Spain). Thus, fractions of 2 ml of 0.1% TFA in 20% ACN, 0.1% TFA in 50% ACN, 0.1% TFA in 80% ACN and finally 0.1% TFA in pure ACN were eluted in this order through the columns. Analyses of the recovered peptides present in the unretained, washing and eluting fractions were carried out by injecting 100 μl of every eluting fraction into the RP-HPLC system as indicated below. The recovery of peptides in every collected fraction was calculated by comparison with the peptide peak areas obtained from the sample reference itself that had been chromatographed prior to SPE.

(b) A second sample of synthetic peptides was obtained by dissolving peptides in acetonitrile–10 m*M* ammonium acetate buffer (pH 5.5) (20:80) to provide a peptide concentration of 0.01–0.1 mg/ml. Portions of 2 ml of this solution were eluted slowly (in duplicate) through the Accel QMA and Accell CM Sep-Pak disposable cartridges connected in series, with the CM connected ahead of the QMA cartridges as described previously [13]. The unretained fraction (2 ml) was recovered (first neutral fraction) and subsequently the cartridges were eluted with 2 ml of 10 m*M* ammonium acetate buffer (pH 5.5) containing 20% of acetonitrile (second neutral fraction). The CM and QMA cartridges were disconnected. Subsequently, portions of 4 ml of 10 m*M* ammonium acetate buffer (pH 5.5) containing 20% of acetonitrile plus 1 *M* sodium chloride were passed slowly through the CM cartridge to obtain the basic fraction and through the QMA cartridge to obtain the corresponding acidic fraction.

(c) Another sample of synthetic peptides was dissolved in 10 m*M* ammonium acetate buffer (pH 5.5) to provide a peptide concentration of 0.01–0.1 mg/ml. Portions of 2 ml of the solution of synthetic peptides dissolved in ammonium acetate buffer (pH 5.5) were loaded on to CBA columns (in duplicate). The unretained fraction was recovered (first fraction). Subsequently, the column was washed with 2 ml of ammonium acetate buffer (pH 5.5) (second fraction) and

elution was carried out with two portions of 2 ml of 0.1% TFA in 40% acetonitrile (third and fourth fractions).

RP-HPLC analyses of 100 μl of every collected fraction from the SPE sorbents (CBA, CM, QMA) were carried out to calculate the percentage recovery of each peptide, as indicated above.

2.4. SPE of peptides from enzymatic hydrolysates

Aliquots (2 ml, ca. 150 nmol) of the casein enzymatic hydrolysates were treated as mentioned above for synthetic peptides to achieve the fractionation of casein peptides in non-polar SPE. In ionic SPE, the enzymatic casein hydrolysates were previously lyophilized and dissolved in 2 ml of acetonitrile–10 m*M* ammonium acetate buffer (pH 5.5) (20:80) previously to start the SPE procedure.

2.5. Concentration, enrichment and fractionation of casein peptides by SPE

A narrower fractionation of peptides from casein hydrolysates was carried out following an increase from 10% to 40% of acetonitrile while increasing 5% of acetonitrile in each fraction. On the other hand, enrichment and concentration of peptides were carried out after diluting the enzymatic hydrolysate 50-fold, so that the volumes of 50 ml of that hydrolysate were eluted slowly (1 ml/min) using a peristaltic pump through the SPE column. The rest of the procedure for elution was as mentioned above.

2.6. RP-HPLC separation of peptides

Experimental work was performed in a Waters high-performance liquid chromatograph controlled with a Maxima 820 workstation (Waters, Milford, MA, USA). It was equipped with two M 6000A pumps, an M WISP 710B automatic sample injector and a variable-wavelength absorbance detector (M 481 L spectrophotometer).

Peptide analysis was carried out using a reversed-phase C_{18} Nova-Pak column (150 mm × 3.9 mm I.D.) (Waters). This column provides a good chromatographic separation of peptides [7]. The chromatographic conditions were as follows: eluent A, 0.1% TFA in water and eluent B, 0.1% TFA in acetonitrile; linear gradient from 0% B to 40% B in 70 min, then at 73 min 100% B and at 78 min 100% A (12 min); flow-rate, 1 ml/min; and absorbance detection at 214 nm. Synthetic peptide injections ranged from 2 to 20 nmol and were analysed at least in duplicate. The amount of peptide injected did not overload the HPLC column and the peak areas were measured using a Maxima 820 workstation.

3. Results and discussion

3.1. Isolation and preparative fractionation of peptides using non-polar SPE

The behaviour of preparative non-polar SPE of peptides was first studied with synthetic peptides loaded on to different commercial non-polar bonded silica disposable columns and cartridges. Fig. 1 shows the RP-HPLC of the solution of ten synthetic peptides used as reference for SPE. Peptides were separated with good resolution for an accurate measurement of peak areas. The average residual standard deviation (R.S.D.) for peak areas after five repetitive injections was less than 1.3%). Peptides loaded on to non-polar SPE units were recovered into six different fractions, i.e., the unretained fraction and the fractions corresponding to eluting the column with 0.1% of TFA in Milli-Q-purified water (washing fraction) and 0.1% of TFA in 20, 50, 80 and 100% of acetonitrile. SPE was carried out using TFA that provides an acidic pH and avoids any peptide ionic interaction with negatively charged silanols. TFA increases the peptide hydrophobicity because carboxylic groups are protonated whereas basic residues form ion pairs with TFA [10]. Table 1 shows the peptide recoveries for the different bonded silica columns. The total recovery of a given peptide in the fractions (given as the sum of the percentage

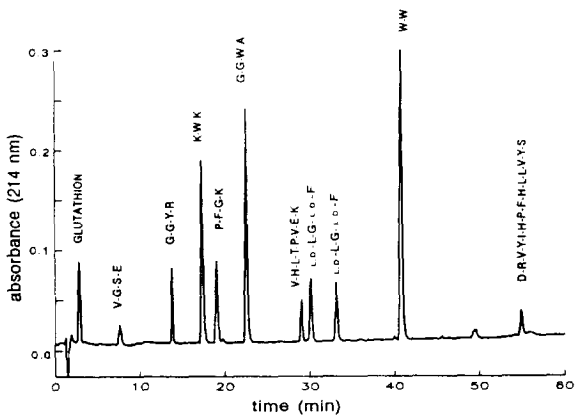

Fig. 1. RP-HPLC of a mixture of synthetic peptides dissolved in 0.1% TFA in water used for studying the selectivity and behaviour of the SPE columns and sorbents. Column, Nova-Pak C_{18} (150 × 3.9 mm I.D.), eluent A, 0.1% (v/v) TFA in water; eluent B, 0.1% (v/v) TFA in acetonitrile; linear gradient from 0 to 40% B in 70 min; flow-rate, 1 ml/min. Duplicate injections of 100 μl were made, using an autosampler. L-D-L-G-L-D-F separates in two diastereoisomeric peaks.

recovery obtained in each fraction) is generally high for most peptides and SPE columns.

Peptides fractionate well in non-polar SPE, depending on their relative polarity or hydrophobicity. Thus, more hydrophobic peptides such as L-G-F, W-W or D-R-V-Y-I-H-P-F-H-L-L-V-Y-S eluted with a higher percentage of acetonitrile, whereas more polar peptides such as glutathione, V-G-S-E or G-G-Y-R could either pass unretained through the SPE sorbent or be eluted in the washing fraction. All peptides elute from the SPE columns with 50% or less of acetonitrile, and no synthetic peptides were recovered with 80% or 100% of acetonitrile. Consequently, the last two fractions are not included in Table 1.

Regarding sorbent selectivity, the comparative study of data of peptide recovery in the eluting fractions exhibits a different retention behaviour between SPE sorbents. Under the experimental conditions employed, a higher hydrophobic interaction between a given peptide and the sorbent is expected to occur when a higher concentration of acetonitrile is needed to elute the peptide from the sorbent. Thus, Table 1 shows that the increasing order of peptide retention because of higher hydrophobic interaction with the sorbent is $CN < C_2 < PH < CH < C_8 < C_{18} <$ Sep-Pak environmental C_{18}. The CN sorbent behaves as a polar sorbent and only allows the retention of more hydrophobic peptides such as W-W and D-R-V-Y-I-H-P-F-H-L-L-V-Y-S. C_2 and PH provide a low retention of the less hydrophobic peptides such as glutathione, V-G-S-E and G-G-Y-R. In contrast, CH, C_8, C_{18} and Sep-Pak C_{18} give a much better preparative isolation and fractionation of peptides. However, C_8 and CH do not retain the very polar peptides sufficiently. In contrast, Sep-Pak C_{18} retains most peptides and is the most suitable when a wide range of peptide polarity and heterogeneity occurs in a sample.

3.2. Isolation and preparative fractionation of casein hydrolysates using non-polar SPE

Further evaluation of peptide SPE was accomplished with a complex mixture of peptides arising from food proteins. Enzymatic hydrolysates of α and β-caseins are used for preparative fractionation and extraction of peptides with non-polar SPE disposable columns. Fig. 2 shows the RP-HPLC of the enzymatic hydrolysate obtained from α-casein. The chromatogram shows a higher number of peptides than those expected from a complete tryptic hydrolysate of α-S1-casein. The reason for this is the existence of an additional activity of chymotrypsin and the fact that bovine α-casein (Sigma) is not a single and pure genetic variant. Nevertheless, the complex mixture of casein peptides obtained is adequate for testing the SPE procedures. Peptides were isolated by SPE and further chromatographed by RP-HPLC, as reported above for synthetic peptides. Table 2 lists the ranges of peptide retention times and peptide recoveries obtained after analysis by RP-HPLC of the peptides isolated from each SPE column and eluting fraction. The peptide recovery in each column and fraction was obtained by comparing the chromatograms of the eluting fractions with the chromatogram of the complete enzymatic hydrolysate before peptide loading into the SPE

Table 1
Fractionation and recovery obtained from a mixture of synthetic peptides in CN, C_2, PH, CH, C_8, C_{18} and Sep-Pak Plus C_{18} column

Peptide	Recovery in the fractions[a] (%)											
	CN column				C_2 column				PH column			
	1	2	3	4	1	2	3	4	1	2	3	4
Glutathione	70	30	0	0	66	39	0	0	53	47	0	0
V-G-S-E	66	26	0	0	56	51	0	0	35	59	0	0
G-G-Y-R	65	32	0	0	18	57	0	0	3	71	22	0
K-W-K	61	32	0	0	16	42	5	0	6	68	18	0
P-F-G-K	66	35	0	0	0	68	22	0	0	0	94	0
G-G-W-A	44	52	0	0	0	12	82	0	0	0	93	0
V-H-L-T-P-V-E-K	55	33	10	0	0	7	72	0	0	0	92	0
L,D-L-G-L,D-F	42	57	2	0	0	0	86	9	0	0	36	71
L,D-L-G-L,D-F	35	58	3	0	0	0	60	13	0	0	20	73
W-W	0	6	86	2	0	0	0	99	0	0	0	100
D-R-V-Y-I-H-P-F-H-L-L-V-Y-S	0	0	58	39	0	0	0	72	0	0	0	108

	CH column				C_8 column				C_{18} column				Sep-Pak Plus C_{18}			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Glutathione	41	58	0	0	33	68	0	0	25	76	0	0	0	7	92	0
V-G-S-E	0	83	0	0	0	33	18	0	0	0	15	0	0	0	85	0
G-G-Y-R	0	5	80	0	0	0	98	0	0	0	101	0	0	0	96	0
K-W-K	0	14	61	0	0	0	95	0	0	0	99	0	0	0	62	19
P-F-G-K	0	0	99	0	0	0	100	0	0	0	101	0	0	0	70	33
G-G-W-A	0	0	91	2	0	0	94	0	0	0	101	0	0	0	2	61
V-H-L-T-P-V-E-K	0	0	86	0	0	0	96	0	0	0	94	0	0	0	27	70
L,D-L-G-L,D-F	0	0	0	96	0	0	10	93	0	0	44	62	0	0	0	99
L,D-L-G-L,D-F	0	0	0	100	0	0	1	99	0	0	14	88	0	0	0	100
W-W	0	0	0	101	0	0	0	103	0	0	0	100	0	0	0	97
D-R-V-Y-I-H-P-F-H-L-L-V-Y-S	0	0	0	96	0	0	0	101	0	0	0	110	0	0	0	103

[a] Fractionation of peptides was carried out as described in the Experimental section. After loading the solution of peptides, four fractions were recovered: (1) unretained fraction; (2) 0.1% TFA in water (washing fraction; (3) 0.1% TFA in 20% acetonitrile; and (4) 0.1% TFA in 50% acetonitrile. Collection fractions of 0.1% TFA in 80% and 100% of acetonitrile did not recover any amount of peptide (result not shown). Data for recovery were obtained in duplicate.

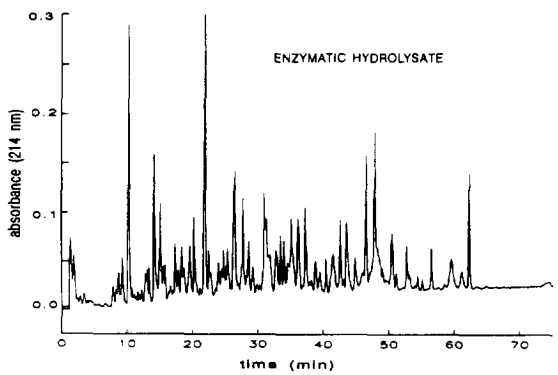

Fig. 2. RP-HPLC of the α-casein enzymatic hydrolysate (Trypsin II, Sigma) used for studying the selectivity and behaviour of the SPE columns and sorbents. Column and conditions as in Fig. 1.

column (Fig. 2). Although Table 2 only shows the results for α-casein, similar relative results were observed for β-casein. As before, peptides from casein distribute into the different SPE fractions, depending on their respective hydrophobicities. In this regard, SPE greatly simplifies peptide samples for further analysis. All peptides elute with 50% or less of acetonitrile, and the total recovery obtained is close to 100% for all columns (the sum of the recovery in each fraction is close to 100% for every column, i.e., the sum of peak areas in all the eluting fractions is equal to the total peak area of the hydrolysate before loading into the SPE column). Therefore, no irreversible adsorption of casein peptides on any of the SPE sorbents seems to occur because all the expected peaks were recovered in the different fractions. Figs. 3 and 4 show the chromatograms of casein peptides isolated using C_2 and C_{18} sorbents. It must be pointed out that no new additional peaks seem to appear in comparison with the chromatogram of the original enzymatic hydrolysate (Fig. 2), which means the absence of artifacts during the SPE procedure.

SPE columns exhibit a similar relative behaviour for the extraction of casein peptides and synthetic peptides. The sorbent's ability to retain peptides by hydrophobic interactions is similar to that observed with synthetic peptides. Thus, Table 2 shows that CN sorbents retain exclusively more hydrophobic peptides (eluting later than 50 min in RP-HPLC and giving a 14% recovery). C_2, PH and CH sorbents behave similarly because they do not retain very polar peptides but provide a good recovery of moderate and very hydrophobic peptides (72, 83 and 83%,

Table 2
Fractionation and recovery of peptides from an enzymatic hydrolysate of α-casein by using different SPE columns

SPE procedure	Fraction in SPE[a]							
	1		2		3		4	
	Δt_r (min)[b]	Recovery (%)[c]	Δt_r (min)	Recovery (%)	Δt_r (min)	Recovery (%)	Δt_r (min)	Recovery (%)
CN	0–46.1	45	0–50.1	45	42.2–59.2	10	52.4–61.9	4
C_2	0–15.1	7	0–24.2	23	19.4–42.2	37	30.6–61.8	35
PH	0–9.8	4	0–15.0	14	14.1–38.4	48	30.8–61.9	35
CH	0–10.1	1	0–14.7	11	12.5–34.7	48	30.6–62.0	35
C_8	–	–	0–10.0	5	9.9–42.1	58	30.8–61.9	36
C_{18}	–	–	0–8.5	2	9.2–38.3	69	30.9–61.8	32
Sep-Pak Plus C_{18}	–	–	–	–	0–27.1	24	10.3–61.8	81

[a] Fractionation of peptides was carried out as described in the Experimental section. The fractions of peptides recovered are as in Table 1.

[b] Range of retention times (min) in which there were found peptides after analysing the collected fractions by RP-HPLC.

[c] Recovery calculated as sum of the total area of the HPLC peaks in the corresponding fraction compared with the total sum of HPLC peaks areas of the complete hydrolysate chromatographed as shown in Fig. 2.

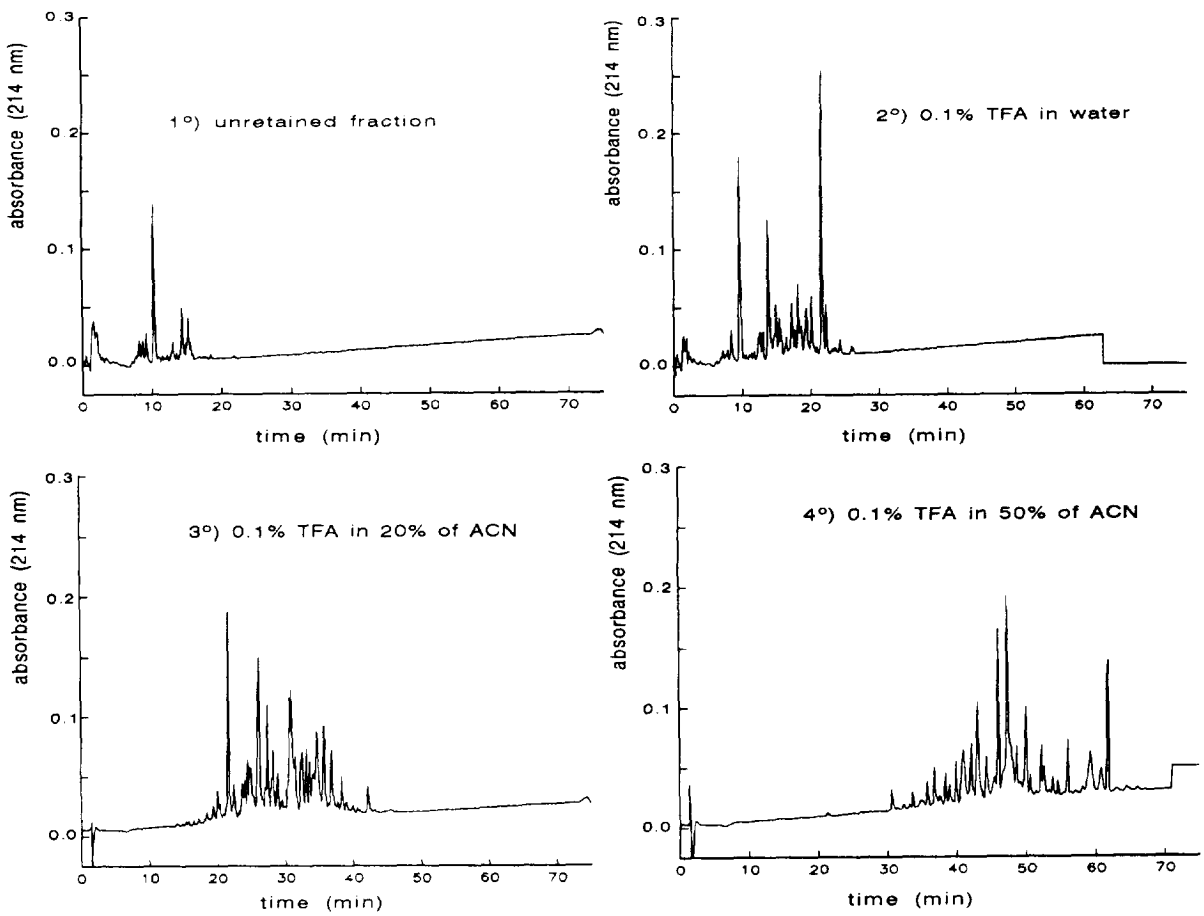

Fig. 3. RP-HPLC of the peptide fractions from an α-casein enzymatic hydrolysate recovered from the C_2 SPE sorbent. (1) Unretained fraction; (2) 0.1% TFA in water; (3) 0.1% TFA in 20% acetonitrile; (4) 0.1% TFA in 50% acetonitrile. Column and conditions as in Fig. 1.

respectively). C_2, PH and CH sorbents also give a good fractionation of the extracted peptides. On the other hand, C_8, C_{18} and Sep-Pak C_{18} retain almost all casein enzymatic peptides with no peptides in the two first fractions (unretained and the washing fractions of 0.1% aqueous TFA).

So far, the results show the suitability of SPE for the preparative extraction and fractionation of peptides. However, a higher preparative purification of peptides may be obtained by small incremental increases in the organic modifier contained in the eluting fractions. Peptides of the casein hydrolysate can be successfully separated into seven fractions from 10% to 40% of acetonitrile. They are collected and subsequently chromatographed by RP-HPLC (Fig. 5). Casein peptides are fractioned as a function of their hydrophobicity, while eluting with an increasing concentration of acetonitrile. In non-polar SPE, peptides adsorb on the sorbent, depending on their hydrophobicity. Thus, more hydrophobic peptides exhibit a higher non-polar interaction with the sorbent and are therefore eluted with a

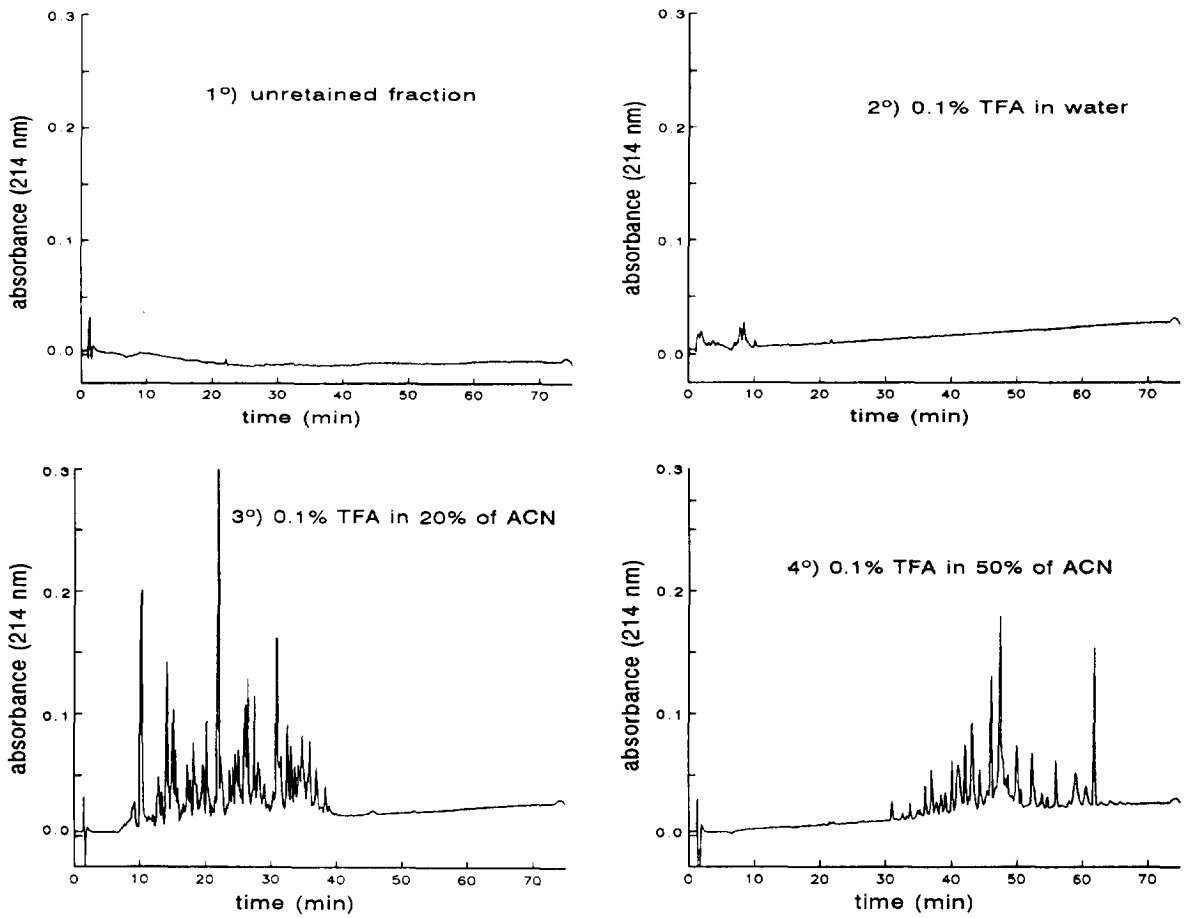

Fig. 4. RP-HPLC of peptide fractions from an α-casein enzymatic hydrolysate recovered from the C_{18} SPE sorbent. (1) Unretained fraction; (2) 0.1% TFA in water; (3) 0.1% TFA in 20% acetonitrile; (4) 0.1% TFA in 50% acetonitrile. Columns and conditions as in Fig. 1.

higher concentration of acetonitrile. In the same manner, those peptides are retained longer in RP-HPLC as expected (Fig. 5).

Selective enrichment and concentration of peptides from diluted casein hydrolysates (1:50) were carried out. After loading 50 ml of a diluted sample of casein peptides, the recovery in the eluting fractions of acetonitrile showed that, except for the CN sorbents, which only adsorb very hydrophobic peptides, giving a recovery of 6%, the rest of the sorbents give a high recovery: C_2 62%, PH 86%, CH 82%, C_8 92%, C_{18} 91% and Sep-Pak Plus C_{18} 99%. These results show that preparative SPE is suitable for the concentration and selective enrichment of trace peptides.

3.3. *Isolation and preparative fractionation of synthetic peptides and protein hydrolysates using ionic SPE*

Peptide isolation and fractionation were also carried out by ion extraction (Table 3). Peptides can be successfully fractionated into basic, neutral and acidic pools using ion-extraction cartridges such as Accell Plus QMA and CM. Table

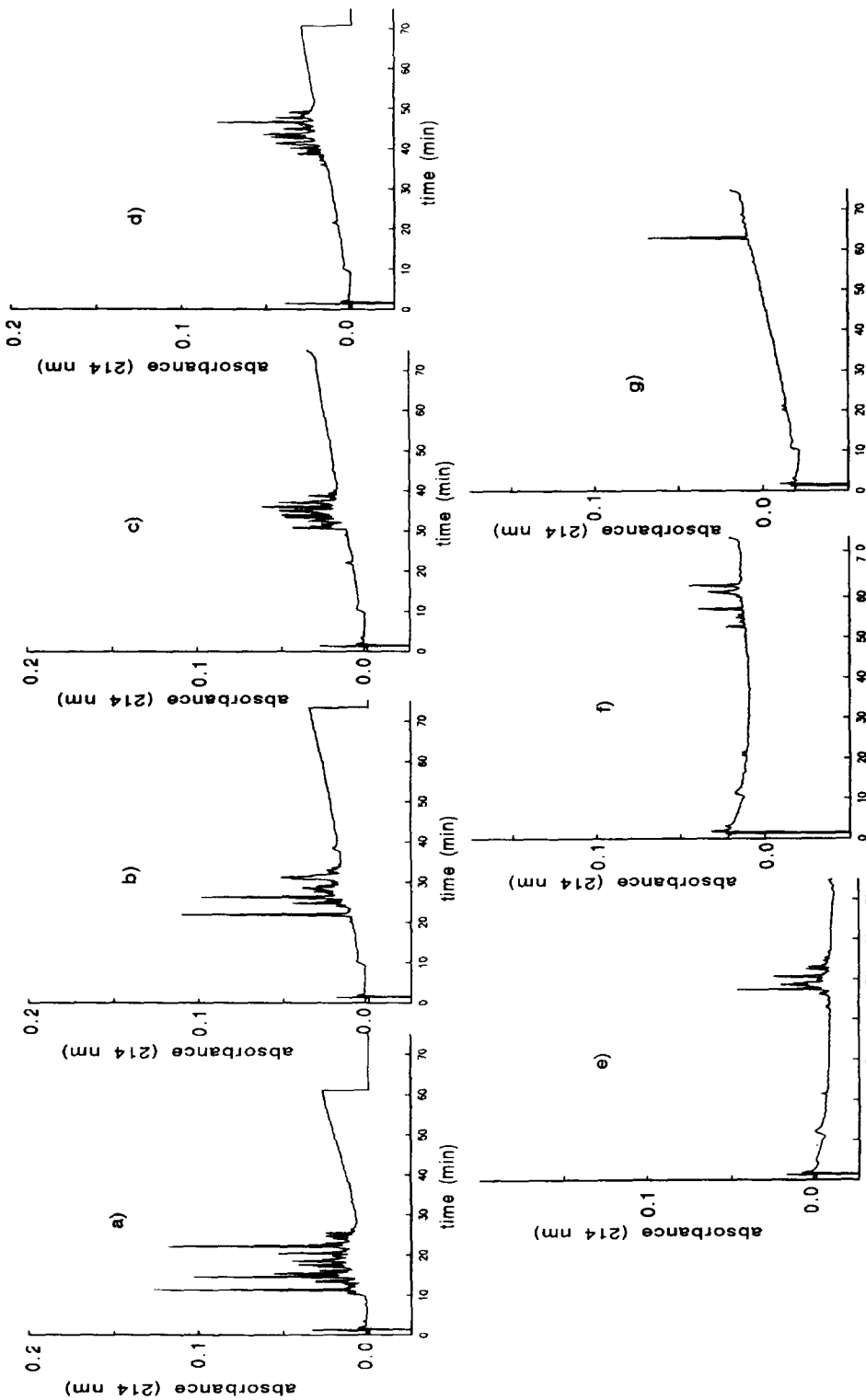

Fig. 5. RP-HPLC of peptide fractions obtained from an α-casein enzymatic hydrolysate after its fractionation in a C_{18} SPE column, using small incremental increases of acetonitrile from 10% to 40%. Fractionation is discussed in the Experimental section. The fractions are (a) 0.1% TFA in 10%, (b) 15%, (c) 20%, (d) 25%, (e) 30%, (f) 35% and (g) 40% of acetonitrile. Column and conditions as in Fig. 1.

Table 3
Fractionation and recovery of peptides from solutions of synthetic peptides in CBA columns and CM and QMA cartridges

Peptide	Recovery in the fractions (%)[a]							
	CBA column				Accell QMA and CM cartridges			
	1	2	3	4	Neutral 1	Neutral 2	Acidic	Basic
Glutathione	46	10	0	0	33	40	25	0
V-G-S-E	87	19	0	0	0	0	80	0
G-G-Y-R	4	33	66	0	0	19	0	65
K-W-K	0	0	85	6	0	0	0	67
P-F-G-K	0	0	100	0	0	29	0	66
G-G-W-A	20	73	1	0	16	83	0	0
V-H-L-T-P-V-E-K	0	0	92	7	4	25	30	37
L,D-L-G-L,D-F	10	82	7	0	21	83	0	0
L,D-L-G-L,D-F	3	75	20	0	19	81	0	0
F-L-E-E-V	39	55	0	0	0	0	98	0
F-L-E-E-I	11	59	30	0	0	0	96	0
F-L-E-E-L	0	50	25	0	0	0	99	0
W-W	0	0	99	1	0	10	88	0
D-R-V-Y-I-H-P-F-H-L-L-V--Y-S	0	0	102	0	0	0	0	96

[a] Fractions are as mentioned in Experimental section. Fractions in CBA are as follows: (1) unretained fraction; (2) 10 m*M* NH_4OAc (pH 5.5); (3) 0.1% TFA in 40% acetonitrile; (4) 0.1% TFA in 40% acetonitrile. Fractions in CM and QMA: neutral 1, unretained fraction; neutral 2, 10 m*M* NH_4OAc (pH 5.5) in 20% acetonitrile; acidic fraction from QMA cartridge, 10 m*M* NH_4OAc (pH 5.5) in 20% acetonitrile + 1 *M* NaCl; basic fraction from CM cartridge, 10 m*M* NH_4OAc (pH 5.5) in 20% acetonitrile + 1 *M* NaCl. Data for recovery were obtained in duplicate.

3 shows that acidic peptides such as F-L-E-E-V, F-L-E-E-I and F-L-E-E-L or V-G-S-E elute in the acidic fraction, whereas G-G-Y-R, P-F-G-K, K-W-K and D-R-V-Y-I-H-P-F-H-L-L-V-Y-S elute in the basic pool. Other peptides appear in the neutral pool. This behaviour agrees with the results reported previously using these cartridges [13].

The basic peptides are efficiently retained in CBA sorbents. At the pH used (5.5), the carboxylic chain of the sorbent is mainly negatively charged whereas peptides with more basic residues (R,K,H) are positively charged and retained on the sorbent. Other sorbents such as SCX (benzenesulphonate), SAX (trimethylaminopropyl) and NH_2 (aminopropyl) were also tested, but the results were poor compared with QMA, CM and CBA and are not included here.

Finally, the enzymatic hydrolysate was also fractioned in the Accell cartridges as shown in Fig. 6. Thus, peptides are extracted into three different pools. From this result, peptides from the α-casein enzymatic hydrolysate seem to appear mainly in the neutral and acidic pools whereas almost no peaks appear in the basic fraction in Fig. 6.

The presence of chromatographic interferences that may co-elute with peptides, possible peptide overlapping, and their presence in very low concentrations are common problems in the HPLC analysis of peptides in food samples and biological fluids. In this regard, SPE should become a necessary preliminary purification step. Peptides can be successfully isolated and further fractioned using appropriate SPE columns and sorbents. The results show the suitability of the SPE procedures based on hydrophobic interaction for preparative extraction, fractionation and enrichment of peptides. However, the correct SPE disposable column must be chosen, depending on the characteristics of the peptides involved. Thus, CN and C_2 sorbents

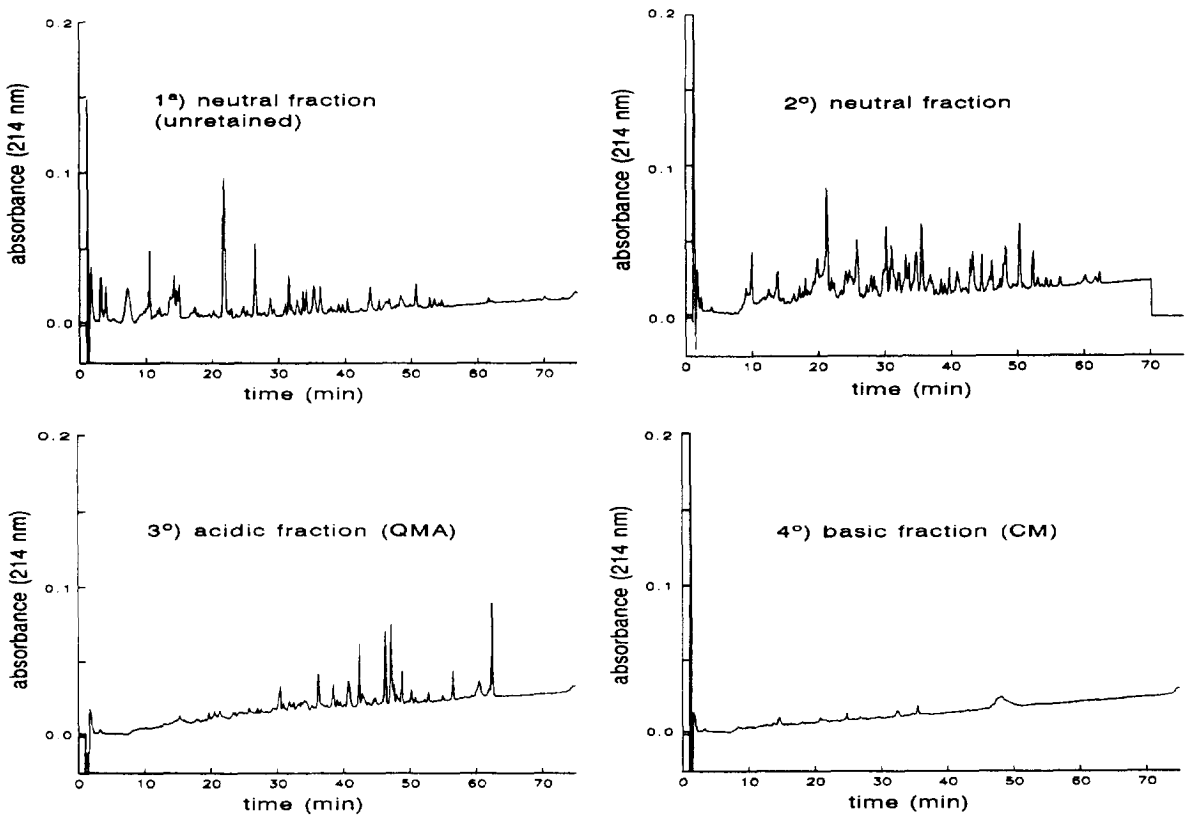

Fig. 6. RP-HPLC of peptide fractions from an α-casein enzymatic hydrolysate recovered from the Accell CM and QMA cartridges. (1) Neutral fraction (unretained); (2) neutral fraction; (3) acidic fraction eluted from QMA cartridge; (4) basic function eluted from CM cartridge. Column and conditions as in Fig. 1.

would be useful for hydrophobic peptides, CH, PH, C_8 for medium and high hydrophobic peptides and C_{18} and Sep-Pak environmental C_{18} for very polar and also hydrophobic peptides. In the non-polar interaction of peptides, as the carbon loading of the SPE column increases and the polarity of the bonded phase decreases, the k' value of peptides increases (i.e., the retention increases). For unknown peptides, it is possible to test several SPE procedures in order to select the most appropriate one or to use non-polar SPE with the highest peptide retention such as a C_{18} packing. Using several SPE procedures could also provide some information on the characteristics of unknown peptides. SPE provides a good recovery and reproducibility in the extraction of peptides, whereas irreversible adsorption is very low and there is an absence of artifacts. This was proved by comparing the HPLC traces from the SPE eluting fractions with those of the initial samples before loading peptides into the SPE column. Using SPE, RP-HPLC mapping of peptides can be simplified and impurities or interferences removed prior to chromatographic analysis. However, it must be pointed out that very large peptides or proteins could be unretained in SPE if they do not pass through the pores of the sorbent. On the other hand, SPE based on ionic interaction could efficiently fractionate peptide mixtures into neutral, acidic and basic pools as shown previously [13]. Ionic SPE would avoid peptide overlapping and improve the resolution while removing peptide interferences present in the sample.

4. Conclusion

SPE is a very useful technique for the preparative isolation, fractionation and purification of peptides from complex mixtures. It provides a rapid and effective way to clean up and concentrate peptides for subsequently RP-HPLC analysis.

Preparative fractionation of peptides requires a correct selection of SPE sorbent, depending on peptide polarity and charge. SPE based on hydrophobic phases is a useful, efficient and rapid procedure for the extraction of peptides after elution with eluents containing an increasing content of acetonitrile in the presence of 0.1% TFA. The increasing order of peptide retention due to the highest hydrophobic interaction between peptides and the sorbent is CN < C_2 < PH < CH < C_8 < C_{18} < Sep-Pak Plus C_{18}. The overall recovery of peptides is high, as proved for synthetic peptides and enzymatic hydrolysates. Concerning sorbent selectivity, it must be pointed out that very polar peptides should be isolated using sorbents with a high retention ability such as C_8, C_{18} or Sep-Pak Plus C_{18}. Very hydrophobic peptides could be isolated with less hydrophobic sorbent such as CN or C_2. Medium and high hydrophobic peptides could be efficiently isolated and fractioned with PH and CH sorbents. SPE with a C_{18} sorbent could be employed for unknown peptides or peptides with a wide range of polarity to provide complete adsorption of most peptides (polar and non-polar). Using several SPE procedures might be useful to provide information on peptide polarity and charge when dealing with unknown peptides. On the other hand, ionic SPE using QMA and CM cartridges simultaneously provides an efficient fractionation and isolation of peptides by charge into neutral, acidic and basic pools as proved for synthetic peptides and casein hydrolysates.

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