Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 105 (2007) 1606–1613
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Prediction of short peptides composition by RP-HPLC coupled to ESI mass spectrometry

M. Schweizer [a], I. Chevalot [a,*], F. Blanchard [a], F. Fournier [a], C. Harscoat-Schiavo [a], R. Vanderesse [b], I. Marc [a]

[a] *Laboratoire des Sciences du Génie Chimique, U.P.R.-C.N.R.S. 6811/I.N.P.L., 13 rue du bois de la Champelle, 54500 Vandœuvre-lès-Nancy, France*
[b] *Laboratoire de Chimie Physique Macromoléculaire, U.M.R.-C.N.R.S. 7568/I.N.P.L., 1 rue Grandville, 54001 Nancy Cedex, France*

Received 14 November 2006; received in revised form 26 February 2007; accepted 19 March 2007

Abstract

The combination of reversed-phase chromatography and electrospray mass spectrometry was used to predict the amino acid composition of low molar weight peptides present in a complex mixture of rapeseed protein hydrolysates. First, amino acid retention times on a hydrophobic stationary phase were evaluated to determine hydrophobicity coefficients for each amino acid and then global hydrophobicity coefficients were established for standard peptides. In this way, a correlation between peptide hydrophobicity coefficients and retention time was established. Then, a C language program was developed to calculate all potential amino acid combinations from the mass of a peptide (molar mass < 1000 Da) and determine theoretical hydrophobicity coefficients corresponding to these combinations. Comparison between the theoretical retention time determined by the model and the experimental retention time resulted in the establishment of a sorted potential composition table. This methodology has been applied to two different rapeseed protein hydrolysates peptides that have been purified and sequenced.

Keywords: RP-HPLC; ESI-MS; Peptides; Protein hydrolysates; Rapeseed

1. Introduction

Peptides, and short peptides in particular, obtained from enzymatic protein hydrolysis are of interest to various industries for their numerous properties. For instance, it has been reported that anti-hypertensive peptides can be found in hydrolysates of food proteins (Pihlanto-Leppälä, 2001) and recently, new anti-hypertensive di- to tetra-peptides have been isolated from rapeseed proteins (Marczak et al., 2003). Furthermore, several studies reported other bioactivities such as antithrombotic (Ariyoshi, 1993), opioid (Zioudrou, Streaty, & Klee, 1979), immunostimulant, antimicrobial (Broekaert et al., 1997), or anti-carcinogenic (Messina & Messina, 1991) properties for different plant protein hydrolysates. The growth promoting effect in cell cultures has been reported for wheat and soy protein hydrolysates (Franek, Hohenwarter, & Katinger, 2000) and for rapeseed protein hydrolysates (Deparis et al., 2003).

Enzymatic hydrolysis of proteins leads to complex mixtures of peptides with different sizes and properties. Peptide sequencing by the Edman method is the most precise tool to determine their amino acid composition but requires peptide isolation and is a very time consuming and expensive method. An alternative to the Edman sequencing method is liquid chromatography coupled to tandem mass spectrometry that has become the most powerfull analytical technique for analyzing peptides. With this combination of techniques, it is possible to isolate precursor peptides and subject them to fragmentation, providing

* Corresponding author. Tel.: +33 383 59 57 95.
E-mail address: Isabelle.Chevalot@ensaia.inpl-nancy.fr (I. Chevalot).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.03.067

the data necessary for peptide sequencing. This method has been used to elucidate the composition of peptides derived from peptic hydrolysis of bovine β-casein, analyzing after their fragment ion spectra (Schmelzer, Schöps, Ulbrich-Hofmann, Neubert, & Raith, 2004). However, these authors pointed out that analyses of low molar mass peptides can result in ambiguous sequence information due to the small number of generated fragment peaks. For a first approach liquid reversed phase chromatography coupled to mass spectrometry could be considered. The use of single mass spectrometry allows the determination of the precise mass but not the sequence of the peptides. However, with the known peptide mass, it is possible to establish a list with all potential amino acid compositions of the considered peptide. The retention time in reversed phase chromatography is directly related to the hydrophobic character of the analysed molecule. Knowledge of both the mass and retention time, should allow improved identification by eliminating proposed compositions that do not satisfy both criteria.

Many authors have described models to predict peptide retention times on a reversed phase column (Guo, Mant, Taneja, & Hodges, 1986; Mant, Burke, Black, & Hodges, 1988; Meek, 1980; Rekker, 1977; Van der Ven, Gruppen, de Bont, & Voragen, 2001; Wilson, Honegger, Stötzel, & Hughes, 1981). However, to the best of our knowledge, no reports have been published that describe the use of information concerning the hydrophobic character for the prediction of the amino acid composition of peptides.

This paper describes an initial stage within a much larger long-term study on peptides from enzymatic hydrolysis. The long-term goal is to find correlations between functional properties/biological activities and hydrophobic/hydrophilic characteristics, charge, amino acid composition and spatial conformation of the peptides. The objective of this work was to develop a method to predict the composition of peptides of low molar weight (<1000 Da) that are present in complex enzymatic hydrolysates using RP-HPLC-ESI-MS analyses and a computer-aided strategy. The knowledge of the amino acid composition of peptides then can indicate some physico-chemical properties and can guide future research regarding functionality or bioactivity of the studied peptides.

2. Experimental

2.1. Preparation of rapeseed protein concentrate and enzymatic hydrolysis

A rapeseed protein concentrate was prepared as reported previously (Deparis et al., 2003) by alkaline extraction followed by isoelectric precipitation. The rapeseed protein hydrolysates were obtained by enzymatic hydrolysis of the protein concentrate. Two enzymes were used separately in this study: Alcalase® 2.4 L (Novo Nordisk, Bagsvaerd, Denmark), a proteinase from *Bacillus licheniformis*, and PTN 3.0 Special® (Novo Nordisk), extracted from pig pancreas. Hydrolysis was performed in a 2 L double wall reactor. The rapeseed concentrate was suspended in 1 L of reversed osmosis (RO) water in order to obtain a protein concentration of 40 g/L. The enzyme to substrate ratio was 1/10. For each hydrolysis, temperature and pH were maintained at the starting values throughout the whole reaction time. The hydrolysis was carried out at 50 °C and pH 8 for PTN 3.0 Special® enzyme and at 60 °C and pH 9 for Alcalase® 2.4 L. The pH control was performed by 0.2 N NaOH addition. Agitation speed was 450 rpm. After 5 h, hydrolysis was stopped by heating the reaction mixture at 95 °C for 10 min. The suspension was centrifuged at 3000*g* for 10 min in order to recover the soluble proteins and peptides.

2.2. Reversed phase chromatography of rapeseed protein hydrolysates coupled to mass spectrometry

Twenty-nine peptides were analysed by reversed phase chromatography coupled to mass spectrometry, using commercially available (Sigma, Saint-Louis, MO, USA) and chemically synthesized peptides. The column used was a C18 Prosphere 300 Å (250 mm × 2.1 mm, Alltech, France). It was kept at 35 °C. Elution was carried out using a linear gradient within 43 min from 100% solvent A (water/acetonitrile/trifluoroacetic (H_2O/ACN/TFA) = 95/5/0.05 (v/v/v)) to 50% solvent B (H_2O/ACN/TFA = 5/95/0.05 (v/v/v)) and 50% solvent A. The flow rate was 0.2 ml/min with UV detection at 214 nm. The HPLC system used was a Perkin–Elmer series 200 system equipped with a UV PE 785A detector (Perkin–Elmer, Norwalk, CT, USA). Eluted peptides were analysed by electrospray mass spectrometry (ESI-MS). The mass spectrometer was a SCIEX API 150EX (PE Sciex, Toronto, Canada). All analyses were carried out in the positive ionisation mode. The following operating parameters were used: mass scan = 100–1500 mass units (m/z), ion spray tension = 5.5 kV, scan time = 1.4 s, step size = 0.2 mass units.

2.3. Peptide sequencing

Two peptides present in the enzymatic hydrolysates were isolated by reversed phase chromatography and submitted to a peptide sequencer. The peptide sequencer was a 476A Applied Biosystems (Applera Corp, Foster City, CA, USA) that uses a PTH RP-18 (2.1 × 220 mm) column (Brownlee, Applera Corp, Foster City, CA, USA).

2.4. Establishment of hydrophobicity coefficients and retention time prediction model

Determination of hydrophobicity coefficients for 20 amino acids was carried out by regression similar to the method described by Meek (1980). The 20 amino acids were eluted separately in RP-HPLC in order to determine their retention times on this hydrophobic stationary phase. Starting values for hydrophobicity coefficients were set at

zero for hydrophilic and neutral amino acids. The starting values for hydrophobic amino acids were determined by their retention time values (in min).

Once the starting values were established, the hydrophobicity coefficients for 29 peptides were calculated by adding the hydrophobicity constants of the amino acids which build the peptide of interest. After this first approach, the peptide retention time was plotted versus the hydrophobicity coefficients and a linear regression was carried out. Since the regression coefficient was not good, the initial coefficients of the amino acids were readjusted in order to obtain a better correlation between retention time and peptide hydrophobicity coefficients. This procedure was applied by iteration with the following global rules: for the four most hydrophobic amino acids (W, F, L, I) the order was kept as initially established, meaning that the optimized coefficients always follow the same pattern than was initially found by the retention time. For neutral and hydrophilic amino acids negative values or zero can be applied depending on the optimization procedure. Repetitive regressions and optimization of amino acid hydrophobicity coefficients were carried out (using standard spread sheet software) until the regression coefficient was satisfying ($R^2 > 0.96$). The final linear regression corresponded to the retention time prediction model used in this study.

2.5. Amino acid residues prediction from the mass of peptides and LC–MS experiments

A C language program was developed to determine the potential amino acid composition of a peptide with a given molar mass. Uncertainty on the experimental mass was accounted for by searching for a peptide mass within a given range (±0.2 mass units). The program calculated all the amino acid combinations and only retained those corresponding to the defined mass range. The core of the program was based on a recursive principle: each "(k)-amino acid" peptide can be used to build 20 "($k + 1$)-amino acid" peptides by condensing one of the 20 elementary amino acids. By using a recursive function, the software can thus build successively all peptides containing 1, 2, 3, ... up to n amino acids (Fig. 1a). The method can be compared to the growth of a tree where each node is a peptide leading to 20 branches. The root therefore yields the first 20 amino acids. In this tree, the generation rank of a peptide corresponds to its number of amino acids. Peptides of higher generations are built by the program in the same way until a predefined generation number, n, has been reached. For computational reasons, the maximum number of amino acids in peptides was limited. At each node, the program calculated the corresponding peptide mass and compared it with the predefined mass range. If there was a match, the peptide was stored in a table without classification. From LC–MS experiments, the experimental retention time of the studied peptide was determined. For each of the predicted amino acid compositions established on the mass criterion, the software calculated the hydrophobicity coefficient and the corresponding theoretical retention time using the previously described model. Then it compared each theoretical value to the experimental one and sorted the propositions. Each studied peptide, i.e. experimental molar mass and retention time, thus led to a single unsorted composition table (based on mass only) and then to a classified table using both mass and retention time (Fig. 1b).

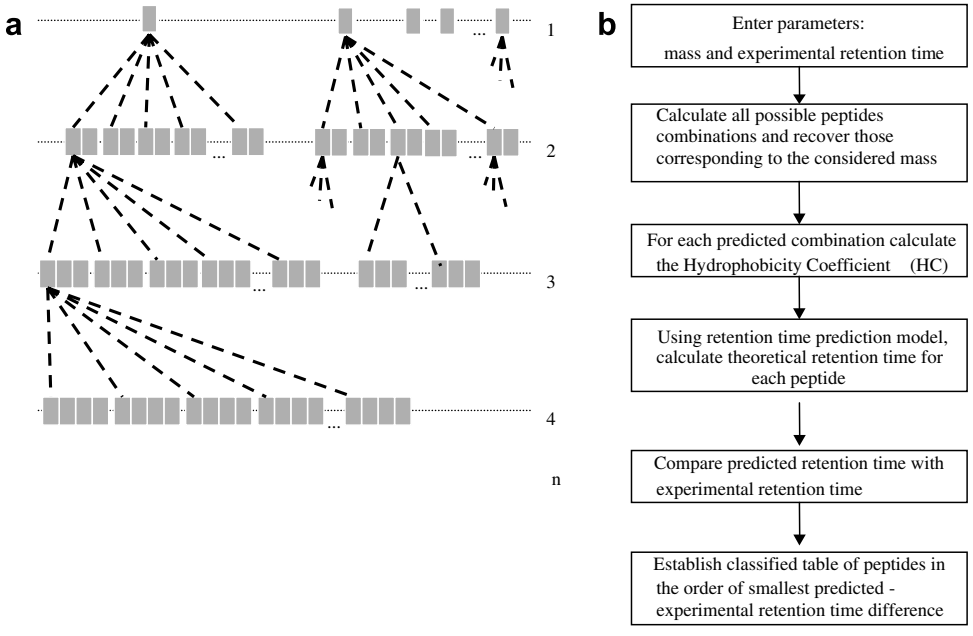

Fig. 1. Description of the amino acid combinations prediction Methodology: (a) systematic peptidic sequences construction by iterative amino acid residue addition and (b) sequential steps used in the program.

3. Results and discussion

3.1. Retention time prediction model

Peptide hydrophobicity coefficients were first determined using amino acid residue coefficients which were reported previously by other authors (Meek, 1980; Van der Ven et al., 2001; Wilson & Honegger, 1981) in order to verify the compatibility of these coefficients with the separation system used in this study. Fig. 2 shows the relations found when plotting retention times of 23 peptides versus the hydrophobicity coefficients calculated by addition of the individual contribution of each amino acid making up the peptide. It appears that none of these models provided a good correlation between the hydrophobicity coefficients of the investigated peptides and their retention times in the separation system used in this study. This result was consistent with previous experimental studies which demonstrated that the apparent hydrophobicity of a peptidic chain was dependent on the properties of the eluent (solvent nature, pH) as well as the chemical characteristics of the non-polar ligand bonded to the chromatographic phase (Wilce, Aguilar, & Hearn, 1995). Therefore, a new set of amino acid hydrophobicity coefficients was determined experimentally (Table 1). Fig. 3 shows that the plot of newly calculated amino acid hydrophobicity coefficients versus retention time for the standard peptides led to two main areas. The peptides with a global theoretical hydrophobicity coefficient inferior to 6, were all eluted with the same retention time of about 5 min. These peptides were hydrophilic low molar weight peptides that were not retained on a reversed phase column. Conversely, for peptides with global hydrophobicity coefficients higher than 6.5, a good correlation was observed ($R^2 = 0.96$), which minimized the degree of error.

In order to test this model of retention time prediction, two peptides with different hydrophobic character were synthesized. These two peptides were *WGNFAVFNGV* ($HC_{theor.} = 36.9$) and *KNFFKE* ($HC_{theor.} = 14.6$). They were eluted on the same separation system and the obtained data were included in Fig. 3 (corresponding to data points 30 and 31, respectively). Excellent agreement between these points and the trend line was observed. This indicated a good predictive accuracy of the new model within the range of hydrophobicity coefficients comprised between 6.5 and 40.

3.2. Prediction of amino acid composition based on peptide mass and hydrophobicity coefficient

Mass spectrometry is widely used to determine the mass of peptides; however many different amino acid combinations can correspond to a given mass (Table 2). All possible peptide combinations were first considered then those corresponding to the molar mass measured by mass spectrometry (within 0.2 unit mass tolerance) were extracted to built a preliminary list of combinations. For instance, for di- and tri-peptides, less than twenty combinations were found corresponding to one mass number with ±0.2 unit mass tolerance. This number of combinations increased dramatically for peptides containing more than 4 amino acids (>500 g/mol) with a thousand of possibilities in the case of hexa and hepta peptides. This list can be sorted by associating a physico-chemical parameter (hydrophobicity) to each peptide and comparing the theoretical retention time value to the observed one obtained by RP-HPLC. This procedure has been applied on some standard peptides (Table 2). Except for peptides exhibiting a large number of potential combinations (superior to one thousand), the first positions in the list were typically found, indicating a good

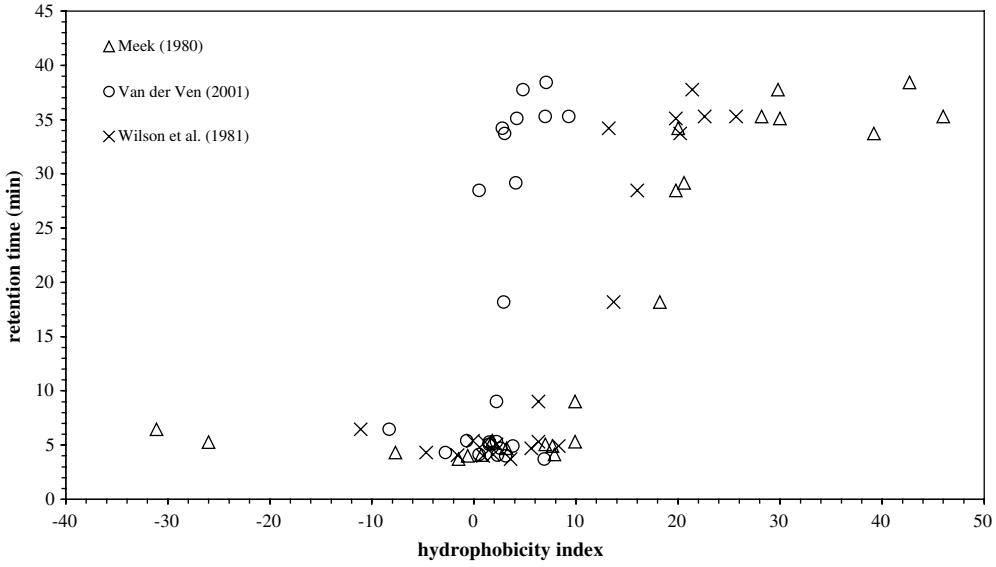

Fig. 2. Correlation of actual retention time on RP column versus peptide coefficients calculated by summing amino acid residues coefficients determined by different authors.

Table 1
Amino acid residues hydrophobicity coefficients determined in this study in comparison with different authors

	Meek (1980)	Wilson and Honnegger (1981)	Van der Ven et al. (2001)	This study
Amide	5	x	x	1.2
Pyroglutamyl	−2.8	x	x	−1.4
W	18.1	7.9	1.8	13.7
F	13.9	7.5	2.6	11.0
L	10	6.6	1.4	8.1
I	11.8	4.3	2.2	5.6
Y	8.2	7.1	1.5	2.1
V	3.3	5.9	1.8	4.2
M	7.1	2.5	0.7	2.5
P	8	2.2	−0.3	0.8
C	x	x	x	−1.0
R	−4.5	−1.1	−1.7	0.1
A	−0.1	−0.3	0.8	0.0
K	−3.2	−3.6	−1.1	−1.3
G	−0.5	1.2	2.3	−1.6
D	−2.8	−1.4	−0.8	2.0
E	−7.5	0	−0.3	−2.9
H	0.8	−1.3	1.5	−1.9
T	1.5	−2.2	−1	0.0
S	−3.7	−0.6	−0.7	−0.6
N	−1.6	−0.2	1	−2.0
Q	−2.5	−0.2	−0.4	−2.2

agreement between the prediction and the experimentally determined amino acid composition of a peptide.

RP-HPLC can provide a quite good prediction of the peptide composition for hydrophobic molecules (hydrophobicity coefficients >6.5) containing two to five amino acid residues. Furthermore for hexa to heptapeptides, the model used can limit the number of combinations to a large extent.

The use of other techniques such as capillary electrophoresis, based on the peptide migration time in a capillary should allow the sorting of the amino acid combinations using a mass-to-charge parameter instead of the hydrophobicity coefficient (Tessier, Blanchard, Vanderesse, Harscoat, & Marc, 2003). This alternate technique would be useful for hydrophilic low molar mass peptides where the use of retention time model from RP-HPLC analysis is not appropriate.

3.3. Application of the methodology on rapeseed hydrolysate peptides

The described methodology was performed on two complex mixtures of unknown peptides from rapeseed protein concentrate (RPC) hydrolyses using two industrial enzymes. Hydrolyses of RPC (with more than 75% protein content) with Alcalase® 2.4 L and PTN® 3.0. resulted in mixtures of 97% and 90% short peptides (<10 kDa), respectively.

Fig. 4 shows the chromatogram for Alcalase® 2.4 L hydrolysate on reversed phase. A large number of peptide peaks were observed and in order to predict the amino acid residues composition of the separated peptides the newly developed software was applied. One peptide from each hydrolysate was isolated manually by pooling together peak fractions of several elutions using HPLC-RP and it was subsequently identified completely by classical Edman peptide sequencing. The reason for choosing the two peaks containing these peptides was that those were the most

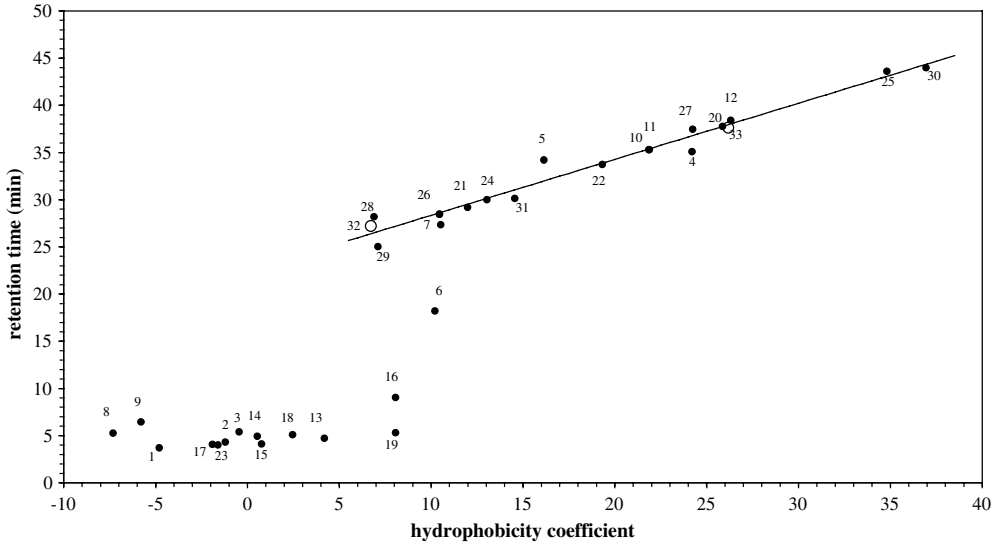

Fig. 3. Relation between retention time in RP column and peptide hydrophobicity coefficients using amino acid residue contributions determined in this study. Open rings correspond to peptides isolated from hydrolysates and used to be applied to the present model. 1: GGG; 2: RK ; 3: KYK; 4: LLL; 5: LL; 6: LY; 7: ACTH (11–24)-KPVGKKRRPVKVYP; 8: β-interleukin-I-VQGEESNDK; 9: RKRSRKE; 10: ACTH (1–24)-SYSMEHFRWGKPV-GKKRRPVKVYP; 11: Angiotensine I/II (3–8)-VYIHPF; 12: Eledoisine: pEPSYDAFIGLM-NH_2^1; 13: AV; 14 : PA; 15: GY; 16: AL; 17: AH; 18: AM; 19: LA; 20: MRLFV; 21: pEHWSY(pE = pGlu); 22: Bradykinine-RPPGFSPL; 23: AG; 24: PLV; 25: RYVFYFV; 26: VYV; 27: WVYV; 28: TITYEY; 29: TVTYKY; 30: WGNFAVFNGV; 31: KNFFKE; 32: ELPP; 33: WIEFK.

Table 2
Molar mass of standard peptides determined by mass spectrometry and number of amino-acid combinations calculated by the program for observed molar mass with ±0.2 mass unit

Standards	Mass determined by MS	Number of total combinations	Experimental retention time	Theoretical retention time	Deviation to theoretical retention time (%)	Rank in classified list
LL	244.4	4	34.2	31.88	−6.96	1
LY	294.4	3	18.2	23.44	+28.7	1
FF	312.4	2	31.38	35.53	+13.21	1
LW	317.4	21	30.08	35.36	+17.56	3
PGF	319.4	14	17.15	23.31	+23.3	2
PLV	327.6	5	29.98	29.96	−0.06	1
GLY	351.6	9	22.13	11.09	−49.8	2
LLL	357.4	16	35.1	36.86	+5.03	3
VYV	379.4	12	27.33	28.41	+3.96	1
WVYV	565.7	309	37.46	36.88	−1.54	3
MRLFV	664.7	1243	37.77	37.88	+0.29	3
pEHWSY[a]	702.6	1790	29.17	29.29	+0.42	18
TISYDL	710.6	957	32.17	32.57	+1.25	21
VYIHPF	774.8	7779	35.28	35.43	+0.42	33
KNFFKE	811.5	6072	29.55	30.89	+4.55	727

[a] pE = pGlu.

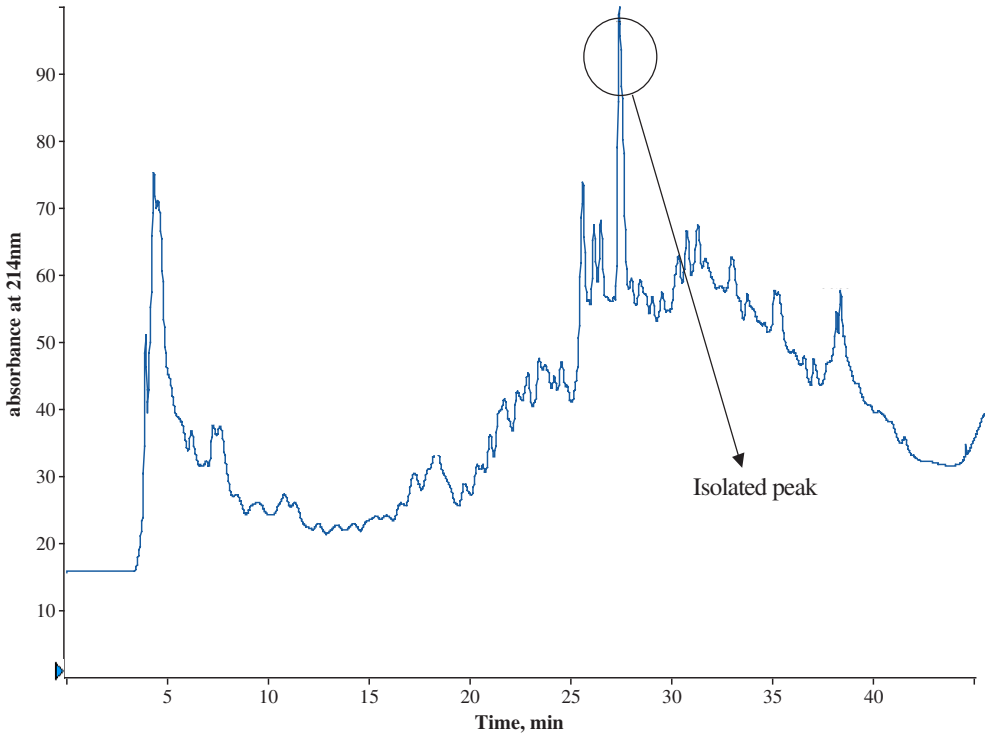

Fig. 4. Reversed phase chromatogram of the Alcalase® 2.4 L hydrolysate. The column used was a C18 Prosphere 300 Å (250 mm × 2.1 mm, *Alltech*). Elution was carried out using a linear gradient from 100% solvent A (H_2O/ACN/TFA = 95/5/0.05 (v/v/v)) to 50% solvent B (H_2O/ACN/TFA = 5/95/0.05 (v/v/v)) and 50% solvent A. The flow rate was 0.2 ml/min.

pertinent peaks and initial mass spectrometry analyses showed a high probability of having only one peptide present within each of these peaks. Sequencing of the isolated peptide from the Alcalase® 2.4 L hydrolysate gave the sequence E-L-P-P (peptide 32 in Fig. 3), whereas the peptide from the PTN®3.0. hydrolysate resulted in W-I-E-F-K sequence (peptide 33 in Fig. 3).

For each isolated peptide, the total number of possible combinations was calculated from the experimental molecular mass. The experimental retention time was compared to the theoretical one and finally the rank of the peptide was pointed out in the classified list (Table 3). It was found that using this new methodology, the actual peptide sequence could be found at the fifth rank for ELPP and

Table 3
Results of peptide sequencing of two isolated rapeseed peptides purified from protein hydrolysates

Peptide sequence	Molar mass (g/mol)	Retention time		Number of potential combinations	Rank
		Experimental	Theoretical		
ELPP	454.52	27.2	26.06	59	5
WIEFK	721.85	37.62	38.08	2497	47

Application to the new identification program.

Table 4
Possible compositions of peptides obtained from rapeseed hydrolysates and identified by mass spectrometry

Retention time (min)	Mass by LC/MS (g/mol)	Number of potential peptides	Identified compositions (position in the protein, native protein name)	Rank
28.43	262.3	4	LM (75–76, cruciferin)	1
			PF (258–259, cruciferin)	2
28.43	487.3	157	PAGPF (38–42 , napin)	1
29.86	657.6	890	LEVIAN (424–429, cruciferin)	4
31.28	434.5	70	FRI (42–44, napin)	1
32.5	495.6	122	SGVSF (41–45, cruciferin)	14
37.62	721.85	2497	WIEFK (398–402, cruciferin)	47

The sequences of rapeseed proteins were obtained from a protein database (PubMed: http://www.ncbi.nlm.nih.gov). A software was used to determine the amino acid sequence from a precise mass into these proteins (http://www.expasy.org/tools/findpept.html).

WIEFK was predicted at rank 47 out of 2497 potential combinations. The retention time deviation for peptide ELPP was 3.9% whereas peptide WIEFK had only a 1.2% deviation. Compared with the total number of proposed combinations that are possible for the determined molar mass of the peptides, it appeared that the procedure offered a significant improvement in the peptide composition prediction.

3.4. Application of the prediction methodology to hydrolysates of proteins with known sequence

The developed prediction strategy was applied to several other peptides derived from hydrolysates that were identified by liquid chromatography coupled to mass spectrometry. The classified list of predicted peptides determined from the molar mass and the retention time was compared to the sequence of rapeseed proteins (cruciferin or napin) using available protein databases and a software able to locate a specific peptide within a given protein. Table 4 shows clearly that among potential solutions it was possible to predict peptides that can be present in rapeseed proteins. For example, for the Alcalase® 2.4 L hydrolysate derived peptide with a molar mass of 487.3 g/mol, 157 potential combinations were found initially but the use of the retention time and the prediction strategy led to the identification of PAGPF corresponding to the 38–42 sequence of napin. With this tool it is possible to obtain a better prediction of the peptide compositions. It is also expected to guide researchers towards peculiar activities of the peptides derived from enzymatic hydrolysis of rapeseed proteins. For example, ELPP purified from Alcalase® 2.4 L hydrolysate could present some anti-hypertensive activities as its composition is similar to previously reported anti-hypertensive peptides (Nakamura et al., 1995).

4. Conclusions

The development of a new method to predict the amino acid composition of low molar mass peptides using mass values and hydrophobic properties determined by LC–MS analysis was described in this study. The method was very reliable for relatively hydrophobic peptides up to five amino acids. For hydrophilic peptides, a similar method can be applied using other physico-chemical properties (work in progress). With this computer-aided strategy it is possible to partially characterize a complex mixture of peptides without any preliminary purification. The method was tested with two additional synthesized peptides corresponding to distinct hydrophobicity. It was then applied to two peptides purified from enzymatic hydrolysis of rapeseed proteins. In addition, by sequencing this rapeseed peptide a sequence was found that could potentially exhibit biological activity.

References

Ariyoshi, Y. (1993). Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends in Food Science and Technology, 4*, 139–144.

Broekaert, W. F., Cammue, B. P. A., De Bolle, M. F. C., Thevissen, K., De Samblanx, G. W., & Osborn, R. W. (1997). Antimicrobial peptides from plants. *Critical Reviews in Plant Sciences, 16*, 297–323.

Deparis, V., Durrieu, C., Schweizer, M., Marc, I., Goergen, J. L., Chevalot, I., et al. (2003). Promoting effect of rapeseed proteins and peptides on Sf9 insect cell growth. *Cytotechnology, 42*, 72–75.

Franek, F., Hohenwarter, O., & Katinger, H. (2000). Plant protein hydrolysates: Preparation of defined peptide fractions promoting growth and production in animal cells cultures. *Biotechnology Progress, 16*, 688–692.

Guo, D., Mant, C. T., Taneja, A. K., & Hodges, R. S. (1986). Prediction of peptide retention times in reversed-phase high-performance liquid chromatography II. Correlation of observed and predicted peptide retention times and factors influencing the retention times of peptides. *Journal of Chromatography, 359*, 519–532.

Mant, C. T., Burke, T. W. L., Black, J. A., & Hodges, R. S. (1988). Effect of peptide chain length on peptide retention behavior in reversed-phase chromatography. *Journal of Chromatography, 458*, 193–205.

Marczak, E., Usui, H., Fujita, H., Yang, Y., Yokoo, M., Lipkowski, A., et al. (2003). *Peptides, 24*, 791–798.

Meek, J. L. (1980). Prediction of peptide retention times in high-pressure liquid chromatography on the basis of amino acid composition. *Proceedings of the National Academy of Sciences, 77*, 1632–1636.

Messina, J., & Messina, V. (1991). Increasing use of soyfoods and their potential role in cancer prevention. *Journal of the American Dietetic Association, 91*, 836–840.

Nakamura, T., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *Journal of Dairy Science, 78*, 777–783.

Pihlanto-Leppälä, A. (2001). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in Food Science and Technology, 11*, 347–356.

Rekker, R. F. (1977). *The hydrophobic Fragmental Constant*. Amsterdam: Elsevier.

Schmelzer, C. H., Schöps, E., Ulbrich-Hofmann, R., Neubert, R. H. H., & Raith, K. (2004). Mass spectrometric characterization of peptides derived by peptic cleavage of bovine β-casein. *Journal of Chromatography A, 1055*, 87–92.

Tessier, B., Blanchard, F., Vanderesse, R., Harscoat, C., & Marc, I. J. (2003). Chromatography applicability of predictive models to the peptide mobility analysis by capillary electrophoresis–electrospray mass spectrometry. *Journal of Chromatography A, 1024*(1–2), 255–266.

Van der Ven, C., Gruppen, H., de Bont, D. B. A., & Voragen, A. G. J. (2001). Reversed phase and size exclusion chromatography of milk protein hydrolysates: relation between elution from reversed phase column and apparent molecular weight distribution. *International Dairy Journal, 11*, 83–92.

Wilce, M. C. J., Aguilar, M., & Hearn, M. T. W. (1995). Physicochemical basis of amino acid hydrophobicity scales: evaluation of four new scales of amino acid hydrophobicity coefficients derived from RP-HPLC of peptides. *Analytical Chemistry, 67*(7), 1210–1219.

Wilson, K. J., Honegger, A., Stötzel, R. P., & Hughes, G. J. (1981). The behavior of peptides on reverse-phase supports during high-pressure liquid chromatography. *Biochemistry Journal, 199*, 31–41.

Zioudrou, C., Streaty, R. A., & Klee, W. A. (1979). Opioid peptides derived from food proteins. *Journal of Biological Chemistry, 254*, 2446–2449.