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Generation of meat-like flavourings from enzymatic hydrolysates of proteins from *Brassica* sp.

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ABSTRACT

Proteins from *Brassica* sp. were prepared by alkaline extraction followed by acid precipitation. A double-enzyme (As1.398 and Flavourzyme) two-stage hydrolysis was used to hydrolyse *Brassica* sp. proteins, and the hydrolysates were used to generate meat-like flavourings. The effect of processing conditions on the volatile products generated from the thermal reaction between the protein hydrolysates and other additives was studied. The results indicated that temperature and pH influenced not only the number but also the amount of products. Those with the most favourite flavour and the highest volatile amount were generated at 160 °C, pH 4.0, whereas a burnt odour was produced at 180 °C, pH 8.0. Analysis using response surface methodology showed that the interaction of pH and temperature had a significant influence on the total amount of volatile products ($P < 0.01$). GC-MS analysis demonstrated that most of the components in the reaction products occur in food flavourings which had been identified in model systems.

1. Introduction

Brassica sp. ranks third in world oilseed production after soybean and cottonseed and before peanut and sunflower seed. *Brassica* sp. includes the crop widely known as canola as well as the more traditional varieties referred to as rapeseed. Despite the widespread use of these oils, the proteins from the meal have not yet been used in human nutrition despite their high biological value. At least in the traditional varieties, this reflects the relatively high contents of undesirable non-protein compounds – glucosinolates, their breakdown products, phenolics and phytic acid in the meal (Naczk, Amarowicz, Sullivan, & Shahidi, 1998; Schwenke, 1994). A number of processes have been developed to reduce the content of these compounds in the meal, and "double-zero" varieties with a low content both of erucic acid and the nutritionally undesirable glucosinolates have been developed (Naczk & Shahidi, 1989; Shahidi, Gabon, Rubin, & Naczk, 1990; Shahidi & Naczk, 1989; Shahidi, Naczk, Rubin, & Diosady, 1988). However, these protein products still contain levels of glucosinolates and other anti-nutritional factors which are too high to be considered as a suitable protein source for human food.

Brassica sp. protein contains an excellent balance of essential amino acids (Sosulski, 1983), the lysine content of the protein averages approximately 6.0%. In addition, there is approximately 3.0–4.0% of the sulphur-containing amino acids, methionine and cysteine, with the proportion of these essential amino acids being close to the desirable levels described by the Food and Agriculture Organisation. So continuous efforts have been applied to develop *Brassica* sp. protein hydrolysate for improvement of functionality and nutritional value (Chabanon, Chevalot, Framboisie, Chenu, & Marc, 2007; Chabanon et al., 2008; Cumby, Zhong, Naczk, & Shahidi, 2008; Pinterits & Arntfield, 2007), or to improve the method of bioactive peptide preparation (Wu, Aluko, & Muir, 2008). Moreover, Canola protein hydrolysates prepared by different enzymes have been found to possess antioxidant activity (Cumby et al., 2008).

In comparison with soy protein, those from *Brassica* sp. can produce more volatile sulphur-containing compounds as a result of thermal reactions (Hurrel, 1982). However, information on *Brassica* sp. protein hydrolysate used as the material to generate "thermal process" flavourings is limited. "Thermal process" flavouring is a comparatively recent term given to a food flavouring which is produced by heating a combination of two or more precursor materials under carefully controlled conditions. The primary reactions occurring in this process are the Maillard reactions. As a result, hydrolysed proteins are useful and important as flavouring agents (Lieske & Konrad, 1994; May, 1991; Swaine, 1993). However, during acid hydrolysis of proteins, undesirable by-products may be formed, including 1-chloro-2,3-propanediol and 1,3-dichloro-2-propanol from vegetable proteins and creatinine in the hydrolysates of meat protein. Accordingly, enzymatic hydrolysis

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of vegetable proteins has the potential to provide a suitable means for preparation of flavourings (Ho, 1989).

In this study the hydrolysates of *Brassica* sp. protein were used to generate flavour substances which were analysed quantitatively and qualitatively. The effects of pH, temperature and degree of hydrolysis of the proteins on the flavour characteristics and the amount of products were investigated. In addition, GC-MS has been applied to the identification and characterisation of the products.

2. Materials and methods

2.1. Enzyme

Neutral Protease As1.398, activity 6.2×10^4 pu/g, was purchased from Genencor International, Wuxi, China and Flavourzyme, activity 500 LAPU/g, was purchased from Novozymes, Bagsvaerd, Denmark.

2.2. Preparation of Brassica sp. protein

Brassica napus seed from an oil processing plant in ZhengZhou, was ground to 80 mesh and defatted with petroleum ether (30–60 °C). The meal was extracted with alkaline solution (0.01 M NaOH, the ratio of meal and water was 1:10 (w/v) for 20 min at room temperature), followed by removal of insoluble residue by centrifugation. The residue was re-extracted with alkali (the ratio of meal and water was 1:5, w/v) and the insoluble residue was removed by centrifugation. The soluble protein was purified by adjustment of the pH with addition of HCl solution and a two-step precipitation process to recover the *Brassica* sp. proteins, first at pH 6.0 then pH 3.6. The precipitated protein was dispersed in water (8%, w/v) and the pH adjusted to 7.0. Mini spray dryer B-290 (BÜCHI Labortechnik AG, Switzerland) was used to dry the slurry with the inlet temperature 160 °C, feed rate 10 ml/min and outlet temperature 80 °C. The protein content of the resultant protein preparation was 71.3% (protein factor 6.25).

2.3. Enzymatic hydrolysis of Brassica sp. protein

Various enzyme treatments were used. Firstly *Brassica* sp. protein was hydrolysed using only As1.398 at 50 °C, pH 7.6, enzyme/substrate ratio (E/S) 3700 (pu/g) (Guo, Zhou, & Gu, 2001), and substrate/solvent ratio ([S]) 10% (w/v). The reaction times used were 1, 2 and 3 h. A further series of hydrolysates was prepared by treating the protein with As1.398 for 3 h, followed by hydrolysis using Flavourzyme at 55 °C, pH 6.2, E/S 37 (LAPU/g), [S] 10% (w/v). The reaction times used for the Flavourzyme treatment were 1, 2 and 3 h (in addition to the 3 h with As1.398) (Cumby et al., 2008). Following this step, the solution was heated to 90 °C for 15 min to inactivate the enzyme. The resultant slurry was centrifuged at 2800*g* for 15 min to remove the insoluble residue. The degree of hydrolysis (DH) was defined as the ratio of free amino groups present in the hydrolysate to the total amount of amino groups in the protein, and the amount of free amino groups was determined by ninhydrin reaction (Guo et al., 2001).

2.4. Preparation of thermal reaction mixture and volatile extraction

A mixture of cysteine (0.1 g) and xylose (1.0 g) was dissolved in 20 ml solution of the enzymatic hydrolysate of *Brassica* sp. protein. The final volume was adjusted to 50 ml with phosphate buffer (0.01 M) at various pH values (4, 6 and 8), then transferred into the reaction vessels and reacted at different temperatures between 100 and 180 °C. After reaction for a period of 2 h and cooling to room temperature, the mixture was extracted twice with dichloromethane, firstly with 100 ml followed by 50 ml. Traces of water in the combined solvent phase were removed by adding sodium sulphate. Solvent extracts were concentrated to a volume of 0.5 ml under a gentle stream of nitrogen gas. Dodecane was added to the concentrate as internal standard prior to GC and GC-MS analysis.

2.5. Gas chromatographic analysis

Gas chromatography was performed using a Shimadzu GC-17A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) fitted with a PEG-20 column (25 m × 0.32 mm id) (Shimadzu, Kyoto, Japan), and a flame ionisation detector (FID) was used to quantify the volatile compounds. The temperature of both injector and detector were set at 200 °C. The oven temperature was maintained for 10 min at 40 °C, then programmed linearly from 40 to 160 °C at 2 °C/min and held at 160 °C for 50 min. A split ratio of 50:1 was used. Samples were analysed in triplicate and volatile amounts were expressed as mg per 100 ml reaction solution (mg/100 ml). The number of chromatographic peaks corresponded to the number of products.

2.6. Response surface methodology

Response surface methodology (RSM) has been increasingly applied to evaluate the influence of individual factors and their interactions. RSM is a statistical technique for designing experiments, developing models, considering the effects of several factors and evaluating optimum conditions for desirable responses. With this approach the interaction of possible influencing parameters on response value can be evaluated with a limited number of planned experiments (Liyana-Pathirana & Shahidi, 2005; Wanasundara & Shahidi, 1996). Therefore, the main objectives of this work were to investigate the interactive effects of pH, temperature and time on the volatile amount and the number of products using RSM. A three level Box–Behnken design with three independent variables was applied for response function fitting (Thompson, 1982) and the corresponding experimental points used are presented in Table 1.

2.7. Sensory tests of the products

In order to evaluate the flavour characteristics of the thermal reaction products, seven panelists were chosen and trained. They were asked to describe the flavour characteristics of the thermal reaction products as non-meat aroma, cooked meat, roasted meat or burnt odour, and also to evaluate the flavour using a hedonic scale as either dislike, like or like strongly.

2.8. Gas chromatography–mass spectrometry (GC–MS) analysis

The thermal reaction products and volatile extracts were prepared following the method described above under different conditions: at pH 4 (100, 120, 140, 160, and 180 °C), pH 6 (100, 120, 140, 160, and 180 °C) and pH 8 (100, 120, 140, 160, and 180 °C). All these volatile products were combined and the mixtures were used for GC-MS analysis. This was performed on a Shimadzu GC-17A gas chromatograph and QP-5000 mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The operation conditions were those described above and mass spectra were obtained by electron ionisation at 70 eV.

3. Results and discussion

3.1. The effects of DH on the products

The enzymatic hydrolysate was prepared according to the method described above. Six samples were hydrolysed for 1, 2, 3, 4, 5 and

Table 1
Box–Behnken three level experimental design arrangement and responses.

Trial no.	Test designs			Results[a]	
	pH X_1	Temperature (°C) X_2	Time (h) X_3	Volatile amount mg/100 ml	Number of products
1	8.0	160	2	43.16	53
2	8.0	120	2	12.84	31
3	4.0	160	2	141.68	61
4	4.0	120	2	7.94	38
5	8.0	140	3	27.92	46
6	8.0	140	1	4.86	35
7	4.0	140	3	49.26	47
8	4.0	140	1	28.88	39
9	6.0	160	3	77.68	63
10	6.0	160	1	40.56	48
11	6.0	120	3	10.94	52
12	6.0	120	1	4.28	43
13	6.0	140	2	26.94	55
14	6.0	140	2	27.70	54
15	6.0	140	2	26.40	55

[a] Samples were analysed by GC in triplicate, using the conditions described in Section 2.5.

6 h and the corresponding DH values were 5.15%, 11.5%, 13.0%, 16.4%, 21.5%, and 26.8% respectively. Each sample was used to generate flavourings. The results indicated that hydrolysates with different DH produced varying quantities of volatile compounds and these increased with increasing DH. On this basis, the hydrolysate with DH of 26.8% was used for further investigation.

3.2. The effect of pH on the products

At relatively low temperatures (100 and 120 °C), the amounts of volatile compounds obtained at pH 8 were more than those at pH 4 and pH 6 (Fig. 1a). On the other hand, at 160 and 180 °C, the amount of volatile compounds at pH 4 was more than those at pH 6 and pH 8. The components of the volatile products were also affected by pH. At 160 and 180 °C, the number of volatile products at pH 4 was more than at pH 6 and pH 8, but at 100, 120 and 140 °C, the number of volatile products at pH 6 were more than at pH 4 and pH 8 (Fig. 1b).

The results showed that Maillard reaction products were strongly affected by pH. The Maillard reaction is usually divided into three stages. The initial stage starts with a condensation between an amino group and a reducing sugar, leading to an N-glycosylamine in the case of an aldose sugar that rearranges into the so-called Amadori product (or Heyns product if the reducing sugar is a ketose). The intermediate stage starts from the Amadori/Heyns product, leading to sugar fragmentation products and release of the amino group. The final stage leads to all kinds of dehydration, fragmentation, cyclisation and polymerisation reactions in which amino groups participate again. Especially in relation to flavour formation, the so-called Strecker degradation is of utmost importance, in which amino acids are degraded by dicarbonyls formed in the Maillard reaction, leading to deamination and decarboxylation of the amino acid. It should also be mentioned that sugar degradation reactions in the absence of amino groups (caramelisation) lead to similar products, but in the Maillard reaction, the amino group acts as a catalyst, so that the Maillard reaction results in a faster reaction and higher amounts of very reactive intermediate products. The various possible reaction paths taking place depend strongly on temperature, pH and nature of the reactants (i.e., type of sugar, type of amino acid, or protein) (Van-Boekel, 2006).

Some research work has also been done with the relationship between pH and volatile products via Maillard reaction. In model system, the overall aroma and the nature of the volatile compounds were all influenced by pH (Meynier & Mottram, 1995). The major products of the methionine systems were dimethyl disulphide and 3-(methylthio) propanal and, as the pH increased, the latter compound showed a small decrease in concentration,

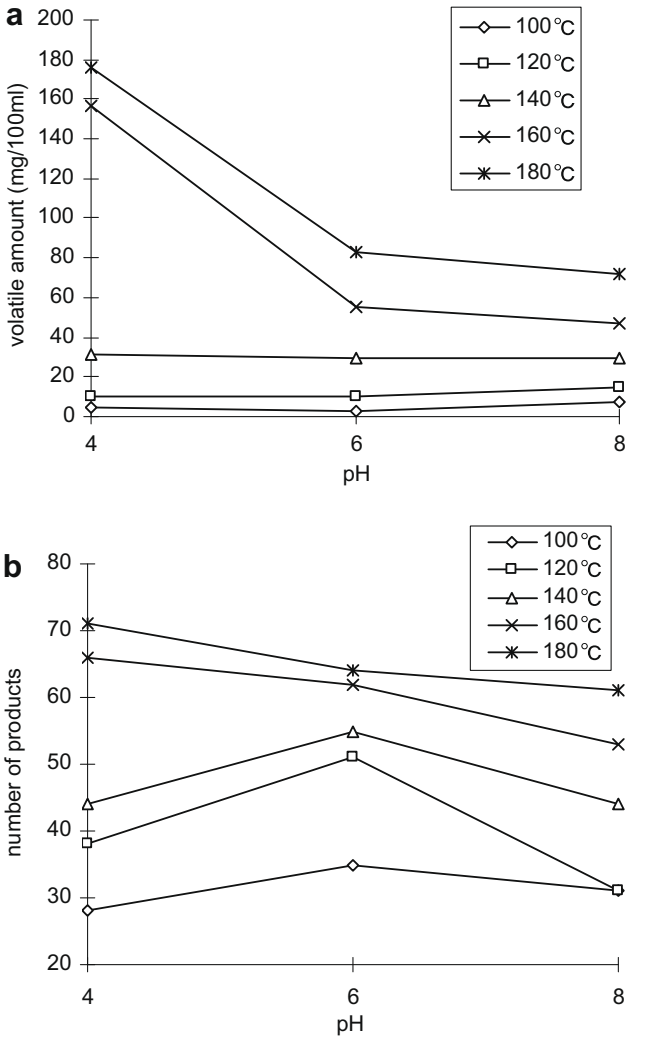


Fig. 1. The effect of pH on the total amount of volatile products (a) and the total number of individual components identified in the products (b) obtained when hydrolysates were reacted with xylose and cysteine for a reaction time of 2 h.

while an increase in the disulphide was observed. The cysteine model system led to a large number of sulphur-containing compounds, including 2-methyl-3-furanthiol, a compound with a strong meaty aroma, whose formation was greatly favoured by lower pH (Meynier & Mottram, 1995). Madruga and Mottram (1995) investigated the effect of pH on the formation of Maillard-derived aroma volatiles using a cooked meat system. The total quantity of volatile compounds increased as the pH decreased. A number of furanthiols and their oxidation products were preferentially formed at acid pH. The formation of other heterocyclic compounds such as thiazoles and pyrazines were favoured by higher pH. Pyrazines are a family of nitrogen-containing heterocyclic compounds. These compounds have important flavour and order characteristics which have a dramatic effect on the sensory aspects of food. Bemis-Young, Huang, and Bernhard (1993) studied the effect of pH on pyrazines formation in glucose–glycine model systems at the broad range of pH value. As the pH increased, the number of pyrazines produced also increased. The greatest varieties of pyrazines were produced at pH 9.0 and 9.64. Martins and van Boekel (2005) researched kinetics of the glucose–glycine Maillard reaction pathways, and the results proved that the initial pH had a different effect on the various rate constants. So the changes in volatiles with pH could be explained by the generation of different concentrations of one or more intermediates in the Maillard reaction. It appears that the protonation of the functional group of the amino acid is also important.

3.3. The effect of temperature on the products

The effect of temperature on the number and amount of the volatile compounds are shown in Fig. 2. The amount of the volatile products all increased with the increasing of temperature at pH 4, 6 and 8. When the temperature was below 140 °C, the amount of the products at pH 4 raised as similar rate as those at pH 6 and 8, but when the temperature was above 140 °C, the amount at pH 4 increased faster than those at pH 6 and pH 8. The number of the volatile products also increased with the temperature increasing at pH 4, 6 and 8. This is expected due to the complicated nature of the reaction paths involved along with the effect of the varied processing conditions. The traditional approach of applying simple kinetics (zero-, first-, or second-order behaviour) is not very helpful because it pertains to only one single step (Van-Boekel, 2006). Again, as expected, the data demonstrate that temperature has an obvious effect on the reaction speed. Generally, the speed is faster at high temperature than at low temperature, so it will produce more products at high temperature.

3.4. The interactive effects of pH, temperature and reaction time

RSM was used to evaluate the interactive effects of reaction pH (X_1), temperature (X_2, °C) and time (X_3, h). Based upon the Box–Behnken three level design for three independent variables, 15 test points were utilised and the results determined for the amount of volatile flavour substances and the number of products are presented in Table 1. Design Expert software V7.1 was used to analyse the test results and the final equation in terms of the factors for amount of volatile material was:

$$\begin{aligned}\text{Volatile amount(mg/100 ml)} &= 176.7175 + 53.016250\,X_1 - 5.726083\,X_2 - 9.710833\,X_3 \\ &\quad + 2.343333\,X_1^2 + 0.037546\,X_2^2 - 8.656667\,X_3^2 \\ &\quad - 0.646375\,X_1X_2 + 0.335000\,X_1X_3 + 0.380250\,X_2X_3\end{aligned}$$

and for number of volatile components was:

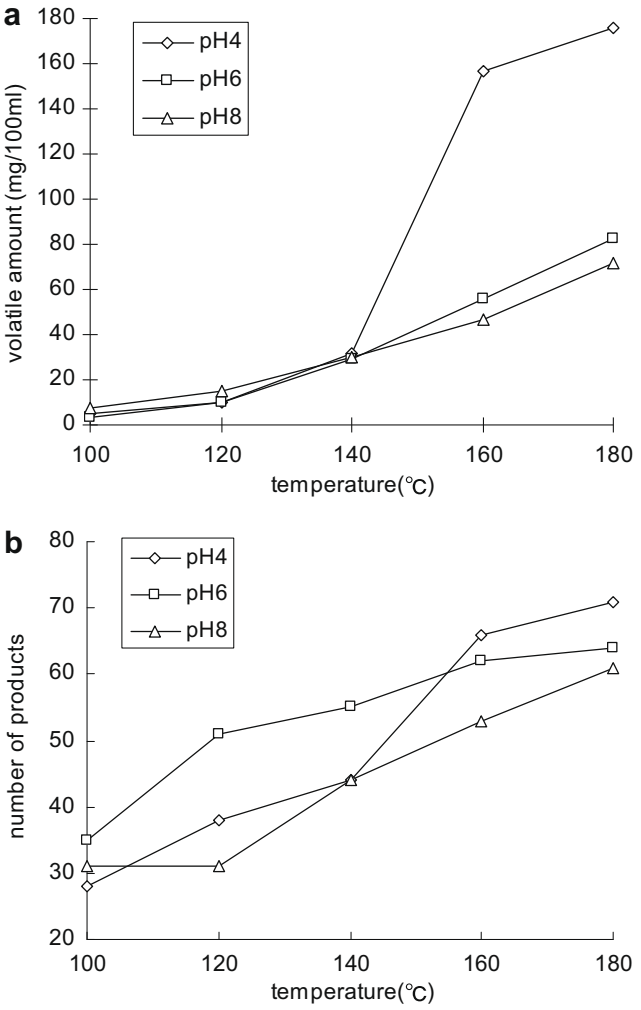


Fig. 2. The effects of temperature on the total amount of volatile products (a) and the total number of individual components identified in the products (b) obtained when hydrolysates were reacted with xylose and cysteine with a reaction time of 2 h.

$$\begin{aligned}\text{Volatile number} &= -59.62500 + 26.87500\,X_1 - 0.022917\,X_2 + 6.95833\,X_3 \\ &\quad - 2.33333\,X_1^2 + (1.04167E-003)\,X_2^2 - 3.58333\,X_3^2 \\ &\quad - (6.25000E-003)\,X_1X_2 + 0.37500\,X_1X_3 + 0.075000\,X_2X_3\end{aligned}$$

ANOVA values for the response surface quadratic model are shown in Table 2. Here the data for the regression demonstrates that the model for volatile amount was highly significant ($P < 0.01$), and that for number of products is significant ($P < 0.05$). The influence of interaction between pH and temperature to volatile amount was also highly significant ($P < 0.01$), whereas the influences of all other interactions were not significant.

3.5. The effect of pH and temperature on the aroma properties of products

The results from the sensory evaluation are presented in Table 3. These show that at the lower temperatures (100 and 120 °C), the products were described as having a cooked meat flavour, above 140 °C, the products had the aroma of roasted meat, whilst at 180 °C, pH 8.0, the products had a burnt odour. These results were corresponding to the food and model system (Meynier &

Table 2
ANOVA for response surface quadratic model.

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*
Volatile amount					
Model	16677.00	9	16677.00	12.23	0.0066
X_1	2414.44	1	2414.44	15.93	0.0104
X_2	8916.48	1	8916.48	58.84	0.0006
X_3	950.92	1	950.92	6.27	0.0542
X_1^2	324.76	1	324.76	2.14	0.2031
X_2^2	832.24	1	832.24	5.49	0.0661
X_3^2	277.00	1	277.00	1.83	0.2343
X_1X_2	2673.92	1	2673.92	17.64	0.0085
X_1X_3	1.80	1	1.80	0.012	0.9176
X_2X_3	231.96	1	231.96	1.53	0.2710
Volatile number					
Model	1116.58	9	124.06	5.11	0.0436
X_1	50.00	1	50.00	2.06	0.2108
X_2	465.13	1	465.13	19.15	0.0072
X_3	231.13	1	231.13	9.52	0.0273
X_1^2	321.64	1	321.64	13.25	0.0149
X_2^2	0.64	1	0.64	0.026	0.8773
X_3^2	47.41	1	47.41	1.95	0.2212
X_1X_2	0.25	1	0.25	0.010	0.9231
X_1X_3	2.25	1	2.25	0.093	0.7731
X_2X_3	9.00	1	9.00	0.37	0.5693

Mottram, 1995; Bemis-Young et al., 1993). The sensory test showed that the most strongly preferred flavour was generated at 160 °C, pH 4.0, and the resultant product had aroma characteristics of roasted meat.

3.6. The compounds identified by GC-MS

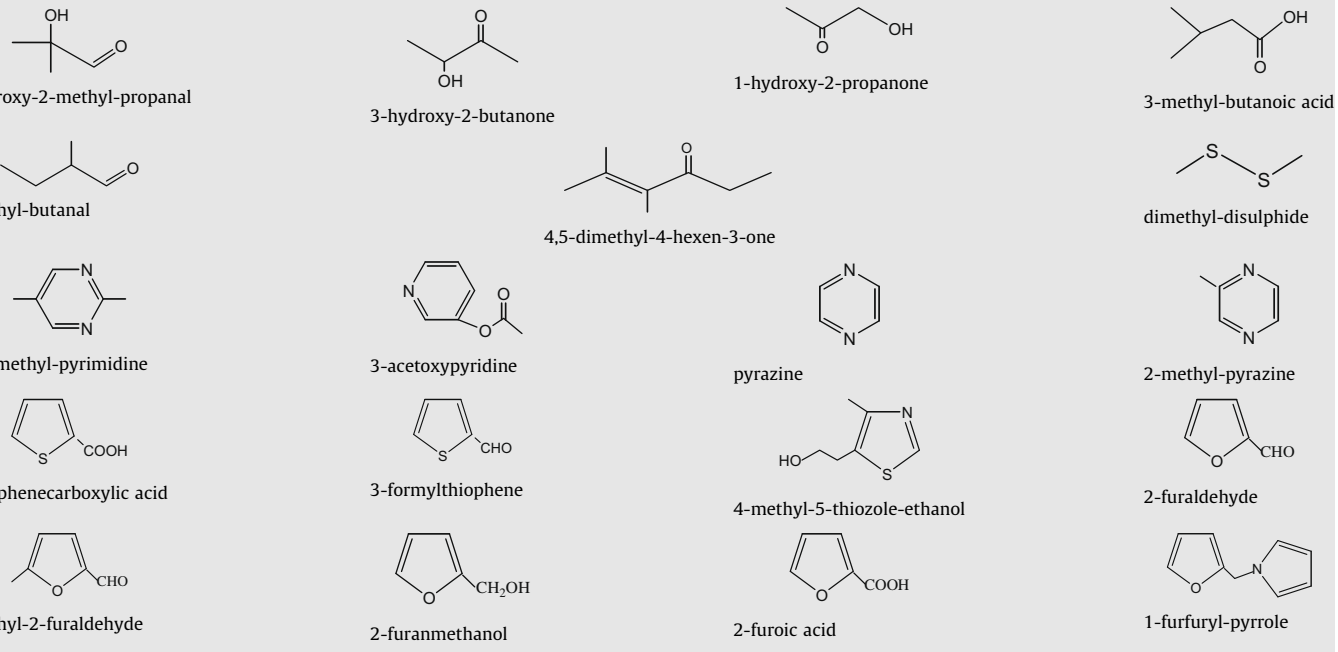
The mixtures of reaction products prepared under different conditions were used for GC-MS analysis. One hundred and thirty two compounds were identified including aldehydes, ketones, acids, multi-sulphur compounds, pyrazines, furans, thiophenes, thiazoles, pyrazoles, pyridines in the dichloromethane-soluble fraction of reaction products, some of these are listed in Table 4. Not all of 132 compounds were found for any one set of reaction conditions and those listed in this table include the major volatile components as well as those which are known to be intermediates in the formation of volatiles.

Most of the compounds found in the reaction products are known to occur naturally in foods and food flavourings (May, 1991). In addition, these have previously been identified in model systems (Apriyantono & Ames, 1993; Bemis-Young et al., 1993). Thus the profiles of flavour compounds produced from *Brassica* protein hydrolysates using the reaction system optimised in the current study were similar to those that might have been expected.

Table 3
Results of sensory evaluation for the flavour characteristics of the thermal reaction products.

Temperature (°C)	pH		
	4	6	8
100	Non-meat aroma, dislike	Cooked meat, dislike	Cooked meat, dislike
120	Cooked meat, like	Cooked meat, like	Cooked meat, like
140	Cooked meat, like	Roasted meat, like	Roasted meat, like
160	Roasted meat, like strongly	Roasted meat, like	Roasted meat, dislike
180	Roasted meat, like	Roasted meat, dislike	Burnt odour, dislike

Table 4
Selected compounds identified in the products generated from *Brassica* sp. protein hydrolysates.[a]



[a] A total of 132 different compounds were identified in the various reaction mixtures by GC-MS analysis. Not all of these were found for any one set of reaction conditions and those listed in this table include the major volatile components as well as those which are known to be intermediates in the formation of volatiles.

Roast flavour is usually associated with the presence of heterocylic compounds particularly pyrazines and thiazoles. Many alkyl pyrazines have been found in meat volatiles (Mottram, 1998). A probable route by which alkylpyrazines might be formed is from the condensation of two α-aminoketone molecules produced in the Strecker degradation of amino acids by dicarbonyl compounds. Alkylthiazoles and alkylthiazolines having low odour threshold values have been found in meat and bread (Elmore & Mottram, 1997). It has also been demonstrated that thiazoles can be formed from mixtures of a hydroxyketone, hydrogen sulphide, ammonia and an aldehyde (Elmore, Mottram, Enser, & Wood, 1997). In the current study, hydroxyketone and aldehyde compounds have been identified in the reaction system (Table 4), and the Strecker degradation of cysteine has previously been reported to produce both hydrogen sulphide and ammonia (Elmore et al., 1997). Furans, furanones, and pyranones compounds have sweet, burnt, pungent, caramel-like ordor, always occur in heated foods, and can be generated from reductones and dehydroreductones (Van-Boekel, 2006).

4. Conclusions

This study clearly shows that the control of pH and temperature is very important during the generation of flavouring following enzymatic hydrolysis of *Brassica* sp. proteins. The formation of meat aroma compound was favoured under lower pH conditions; and at low temperatures the aroma was similar to that of cooked meat and at higher temperatures it was like roasted meat. From the various conditions studied, the most favoured products were generated at 160 °C at pH 4.0., whilst products having a burnt odour were produced at pH 8.0 and 180 °C.

GC-MS analysis demonstrated that most of the compounds in the reaction products occur naturally in foods and have previously been identified in model systems. The enzymatic hydrolysates of *Brassica* sp. protein can be used as the primary ingredient for the production of thermal processing flavours which have meat-like characteristics when evaluated by a sensory panel.

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