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Short communication

Monitoring of enzymatic hydrolysis of penicillin G by pyrolysis-negative ion mass spectrometry

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Abstract

A pyrolysis-negative ion mass spectrometry (Pyr-NIMS) is used for the monitoring of enzymatic hydrolysis of penicillin G (Pen G) to 6-aminopenicillanic acid (6-APA) and phenyl acetic acid (PAA). The high sensitivity and rapid response time of Pyr-NIMS allow its application to the simultaneously determination of these compounds. The mass to charge (m/z) values of 262, 156 and 135 of Pen G, 6-APA and PAA respectively, are used for the quantitative measurements by selected ion monitoring (SIM). The limit of detection (LOD), linearity and relative standard deviation ($n = 5$) are 10 ng ml^{-1}, 100 ng ml^{-1}–1000 mg ml^{-1} and 1.5%, respectively The results are compared with high performance liquid chromatography (HPLC). An important advantage of the presented analytical system is the high linearity of signals without preliminary separation and recalibration. The main and interactive effects of pH, temperature and concentration of Pen G for enzymatic hydrolysis of Pen G are studied. Optimize conditions of pH (8), temperature (28 °C) and concentration of Pen G (12% w/v) in real samples are obtained.

Keywords: Penicillin G; 6-Aminopenicillanic acid; Pyrolysis; Mass spectrometry; High performance liquid chromatography

1. Introduction

6-Aminopenicillanic acid (6-APA) is the common precursor for the synthesis of semi-synthetic penicillins. 6-APA is produced by the hydrolysis of penicillin G (Pen G, benzylpenicillin) or penicillin V by the enzyme penicillin acylase from (*Escherchia coli*, *E-coli*), which hydrolyses the side chain of Pen G to give phenyl acetic acid (PAA) and 6-APA. Quantitative determinations are necessary in all steps of production, isolation, purification and characterization of pharmaceutical final antibiotic products [1–5].

In the case of enzymatic reactions, compounds must be monitored selectively and reproducibly in the processes and this must be done without any (or without time-consuming) separation steps to lower a dead time. In addition, the methods should be stable and should operate without recal-

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ibration during the processes. A large number of methods have been used for monitoring enzymatic hydrolysis of Pen G, such as electrochemical [6–8], spectroscopy [9–12] and chromatographic methods [13–16]. These methods usually require a preliminary separation (e.g. chromatography and spectroscopy methods), or recalibration steps (e.g. electrochemistry) prior to identification and determination of compounds in this enzymatic reaction.

The potential for sensitive analysis and accurate identification has made the mass spectrometric method increasingly attractive for bacterial and enzymatic reactions [17–21]. Pyrolysis is the thermal degradation of complex materials in an inert atmosphere or in vacuum to cleave at a weakest bond to produce smaller and more volatile fragments. Pyrolysis mass spectrometry (Pyr-MS) is an instrument-based technique for rapid, automated and quantitative analysis of biological compounds without preliminary separation and recalibration [22–26].

We have already reported that the determination of cyclosporin A and vancomycin in drug and blood samples were carried out by Pyr-MS [27,28]. Also, we used NIMS for simultaneously determining the herbicide naptalam and its degradation product [29]. In this work, Pyr-NIMS is applied to the simultaneous monitoring of Pen G, 6-APA and PAA.

2. Experimental

2.1. Apparatus and reagents

The HPLC grade methanol was supplied by Merck. Pen G, 6-APA and PAA were obtained from Gist-Brocades. *E-coli* enzyme was purchased from Rosh (PGA 450). All mass spectra were recorded on a VG instrument model Trio 1000. The ion source was operated at 25 eV under a pressure of 4×10^{-4} bars and a temperature of 250 °C.

The liquid chromatography system was equipped with a Waters pump model 510. The UV-spectra of HPLC detector were recorded at wavelengths of $\lambda = 200$–600 nm using a photodiode array detector (PDA).

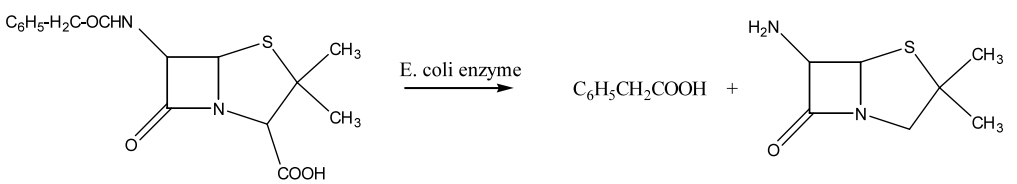
2.2. Determination of enzyme activity

The method is based on the determination of PAA formed during Pen G hydrolysis by NaOH. The enzyme (0.1 g) is suspended in 30 ml of water in the reaction vessel thermostated at 28 °C. After addition of potassium benzylpenicillin (1 g) to the vessel, the pH is adjusted to 8 and kept there by the addition of 0.1 M NaOH by means of the automatic titration equipment with vigorous stirring. A normal time for the analysis was 5–10 min. The activity is calculated according to the following equation.

$$\text{Enzyme activity} = \frac{\text{Titrante volume (ml min}^{-1}\text{) of NaOH} \times 0.1\ \text{mol l}^{-1}}{\text{Weight of enzyme (g)}}$$

2.3. Procedure

The conversion of Pen G to 6-APA was carried out starting with Pen G in a phosphate buffer and



Penicillin G Phenylacetic acid 6-APA

Fig. 1. Pathway of the enzymatic formation of 6-APA.

various conditions of pH, temperature and concentration of Pen G. The immobolized penicillin acylase was added to each solution and the solutions were then equilibrated in a thermostat water bath, controlled to within ± 0.05 K. The solutions were continuously stirred with a Teflon-coated stirrer prior to injection into the HPLC or Pyr-NIMS.

The samples were measured using Waters C18 column μ-Bondapak (4.6 mm × 25 cm) packed with 5-μl particles and a C18 pre-column (39 × 20 mm). The composition of the mobile phase was 29% methanol and 71% v/v phosphate buffer 0.5 M (isocratic elution). The conventional 50 μl sample loop (Rheodyne) was used. The column temperature was maintained at 25 °C. The flow rate and wavelength for quantitative works were set at 1 ml min^{-1} and 225 nm, respectively.

Two solid probes were used. One, a standard heatable probe with a temperature range of 150–600 °C, and the other, a pyrolysis probe made in this laboratory based on the Curie point temperature. Quantitative analyses were performed by SIM method at 262, 156 and 135 m/z for Pen G, 6-APA and PAA. The heating time for the standard heatable probe was 3 s and that of the pyrolysis probe was less than 1 s.

The pyrolysate was bombarded with low energy electron (20 eV) producing both molecular and fragment ions. Prior to pyrolysis, the samples were oven-dried at 50 °C for 30 min. Each sample was analyzed in triplicate.

3. Results and discussion

3.1. Positive and negative ions mass spectrometry

The enzymatic hydrolysis of Pen G to 6-APA is shown in Fig. 1.

When electron impact ionization (25 eV) is used for the determination, many types of positive and negative fragment ions of Pen G, 6-APA and PAA are formed in the ion source. Fig. 2(a–c) represents the positive ion electron impact ionization fragments of pyrolysis of Pen G, 6-APA and PAA, respectively. These spectra do not reveal any characteristic peaks for these compounds. Thus, positive ion electron impact ionization mass spectrometry cannot be utilized for the simultaneous determination of Pen G, 6-APA and PAA.

Fig. 3(a–c) represent typical pyrolysis negative ion spectra of Pen G, 6-APA and PAA, respectively. The spectra of Pen G show the characteristic peaks (113, 137, 151, 262 m/z), of 6-APA (116, 183, 215 m/z) and of PAA (101 and 135 m/z). Therefore, the negative ion characteristic peaks at 262, 156 and 135 for Pen G, 6-APA and PAA, respectively, can be used for the simultaneous identification and determination.

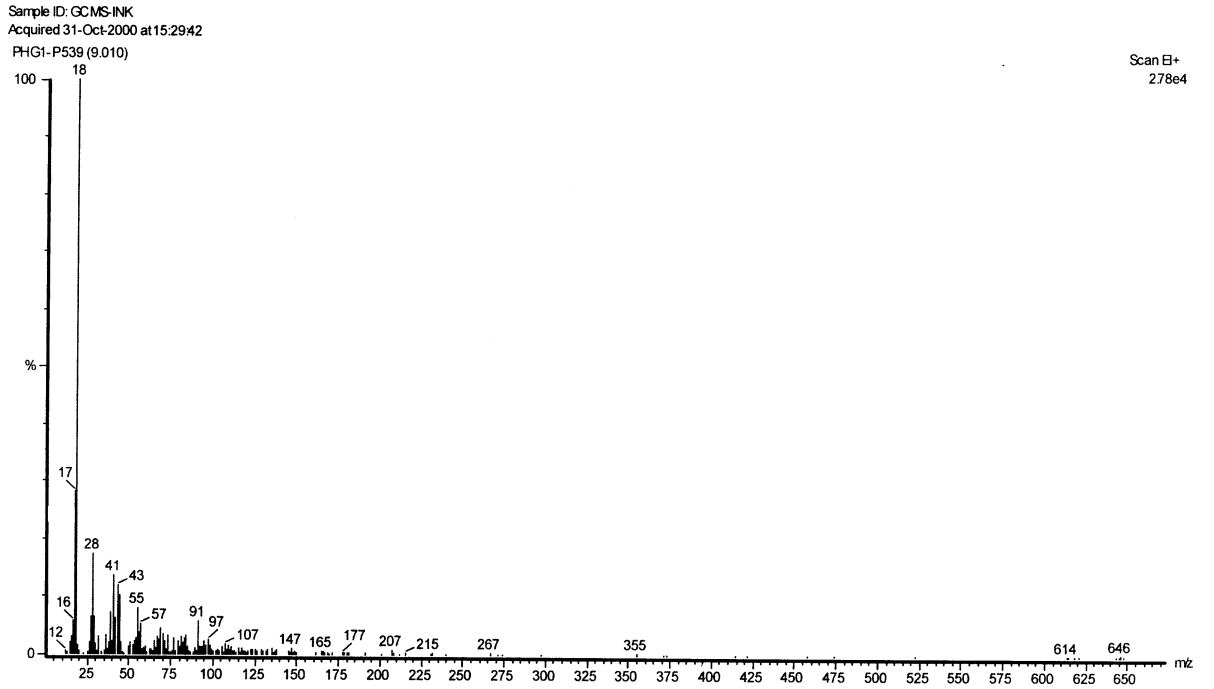
Pyrolysis mass spectral analyses with negative ions are complicated by variation of ion source pressure. A constant flow of an inert gas (He, 0.1 ml min^{-1}) into the ion source, eliminates the variation of ion source pressure.

The temperature of the probe and the ion source are important parameters for the analysis of these compounds and should be optimized. In order to find the best pyrolytic temperature, we examined several temperatures in the range of 150–600 °C, employing the heatable solid probe of MS. The results showed that the mass spectra are reproducible over the temperature range of 350–450 °C.

The major contribution to long-term irreproducibility is ion source aging which alters the transmissivity of ions, thus causing spectral drift. Recently, Goodcare and his groups [30–35] introduced some of chemometric methods for the compensation of drift in quantitative MS data. We noticed that a selection of internal standard compounds (in the present work, 3-nitroaniline) can continuously recalibrate the system and overcome drift problems of Pyr-NIMS data.

The Pyr-NIMS of the Pen G, 6-APA and PAA have peaks at 262 156 and 135, respectively. The SIM method was used for quantitative work at these mass numbers and equation for the calibration curve of Pen G is $I = 40521.71 + 18.8$. Pyr-NIMS can simultaneously determine Pen G, 6-APA and PAA during the enzymatic hydrolysis with average precision and recovery 1.5 and 100.8%, respectively. The standard solution of Pen G is decomposed after 9 h.

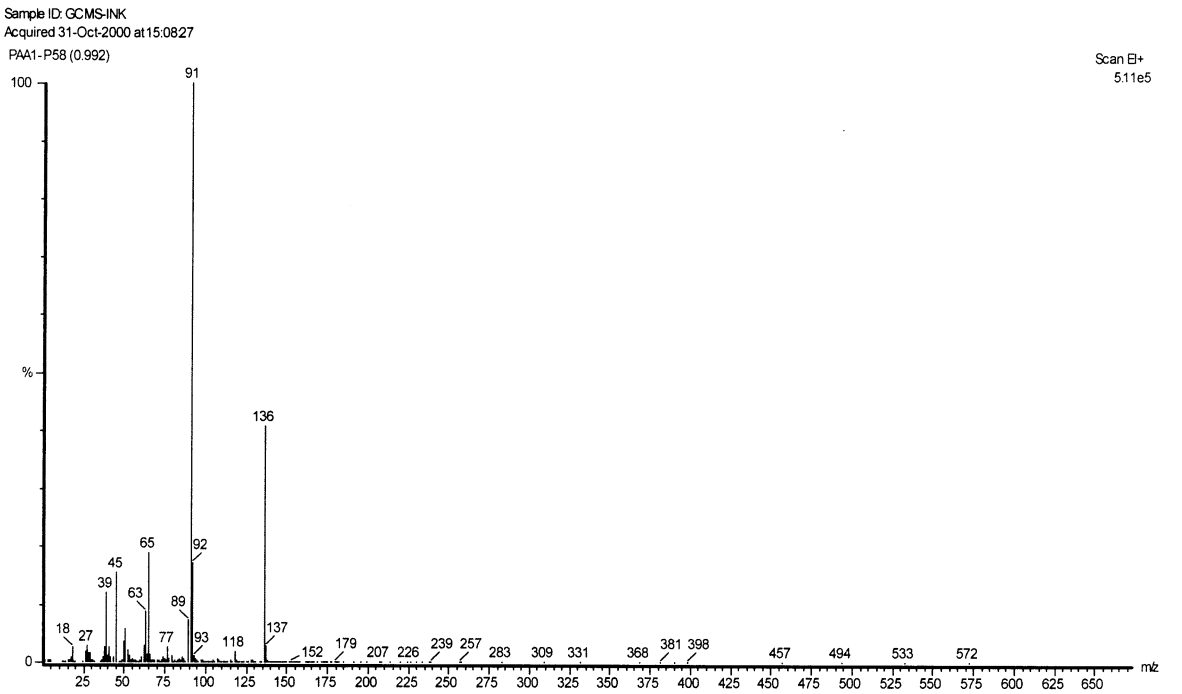
The linear range of Pen G, 6-APA and PAA, under optimized conditions, is 100 ng ml^{-1}–1000



(a)

(b)

Fig. 2. Positive ion mass spectra of Pen G, 6-APA and PAA, respectively.



(c)

Fig. 2. (*Continued*)

mg l^{-1} by normalization method. At the higher concentrations, the signal strongly depends on the concentration and composition (phosphate or borate) of the buffer used.

3.2. High performance liquid chromatography

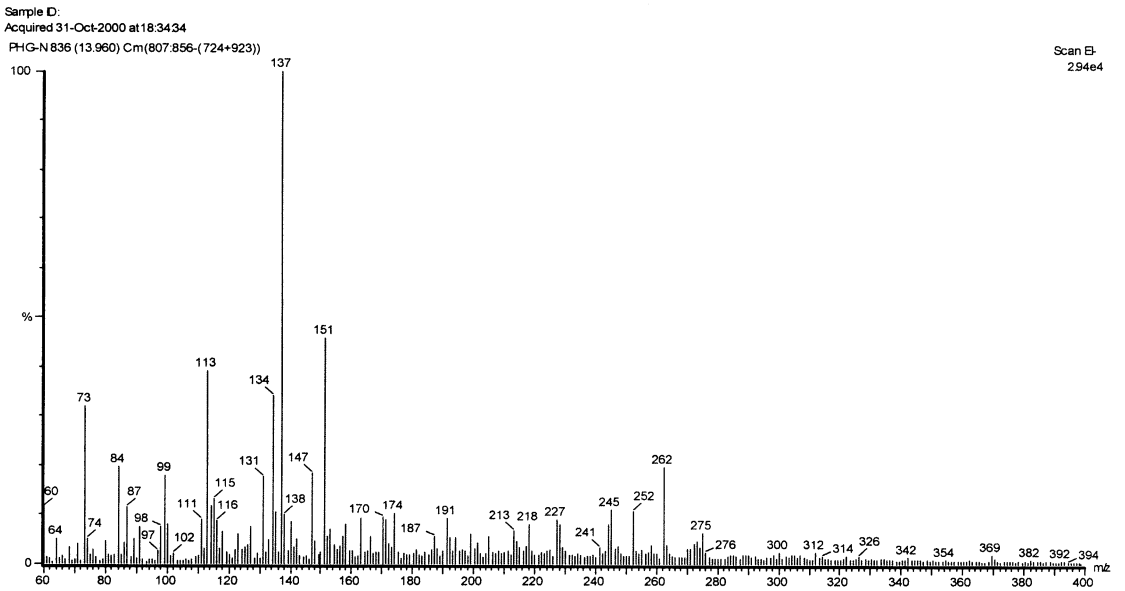
For comparison the analyses were also performed by the HPLC method. As can be seen in Table 1, the LOD, linearity, RSD, correlation coefficient, recovery, sample volume and time of analysis by NIMS are far better than by HPLC.

The enzymatic hydrolysis of conversion of Pen G has also benzylpenicilloic acid as a by-product at pH ≥ 9 at 1.558 min, Fig. 4.

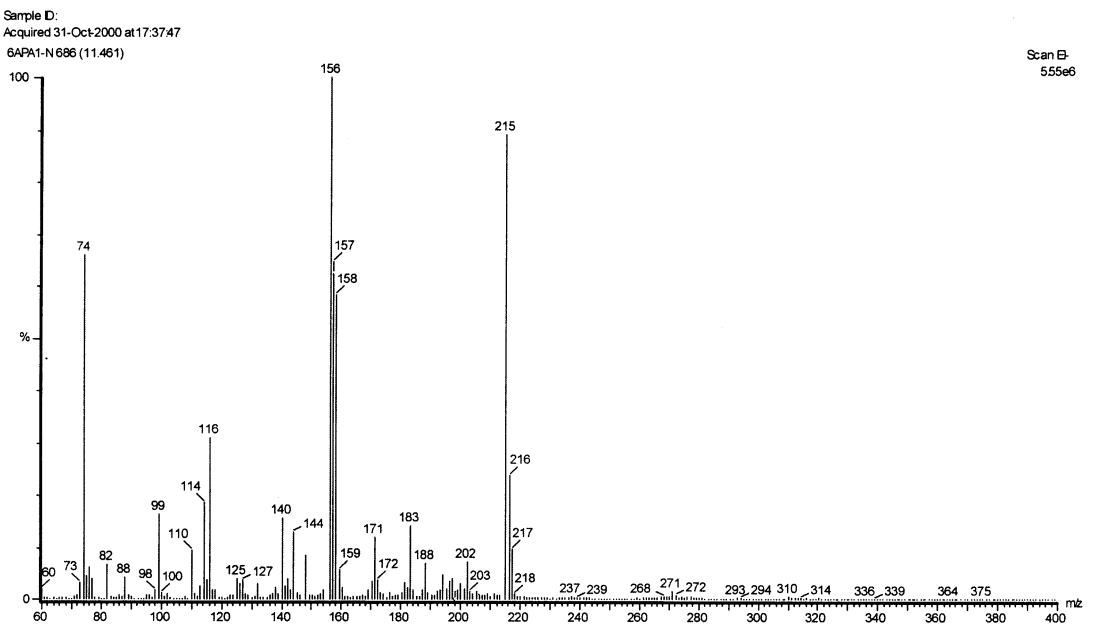
Since benzylpenicilloic acid has also a peak at 156 m/z in Pyr-NIMS spectrum, it can not be determined by this method.

3.3. Factorial design

The effect of several factors (pH, temperature and concentration of Pen G) have been studied. The experimental design approach was employed and a 2^K factorial design was run where 2 stands for a variable level considering the higher and lower values and K is the number of factors studied. The highest and lowest values were determined and assigned as + and − coded levels, respectively, and both are shown in Table 2. For pH, the highest was 9 and the lowest was 6. For temperature the highest was 35 and the lowest was 21. For concentration of Pen G, the highest was 12% and the lowest was 6.5% w/v, respectively. The responses of eight experiments, in Table 2, were obtained based on measurements of SIM (at 156 m/z) intensities of 6-APA.



(a)



(b)

Fig. 3. Negative ion mass spectra (a) Pen G, (b) 6-APA and (c) PAA, respectively.

Table 1
Comparison of Pyr-NIMS and HPLC methods

Characteristic	Pyr-NIMS	HPLC
Mean*	756 ng ml^{-1}	738 ng ml^{-1}
Recovery %	100.8%	98.4%
Correlation coefficient	0.9993	0.9986
RSD**	1.5%	3%
LOD	10 ng ml^{-1}	45 ng ml^{-1}
Performance time	35 min	4 h
Linearity	100 ng ml^{-1}–1000 mg l^{-1}	150–2000 ng ml^{-1}
Sample volume	100 μl	1 ml

* Mean of three spiked (750 ng ml^{-1}) samples.
** Relative standard deviation ($n = 5$).

The results in Table 2 show that the concentration of Pen G has a negative effect on the response. Thus we could not obtain optimal condition by the Simplex method and were obtained with one at a time method.

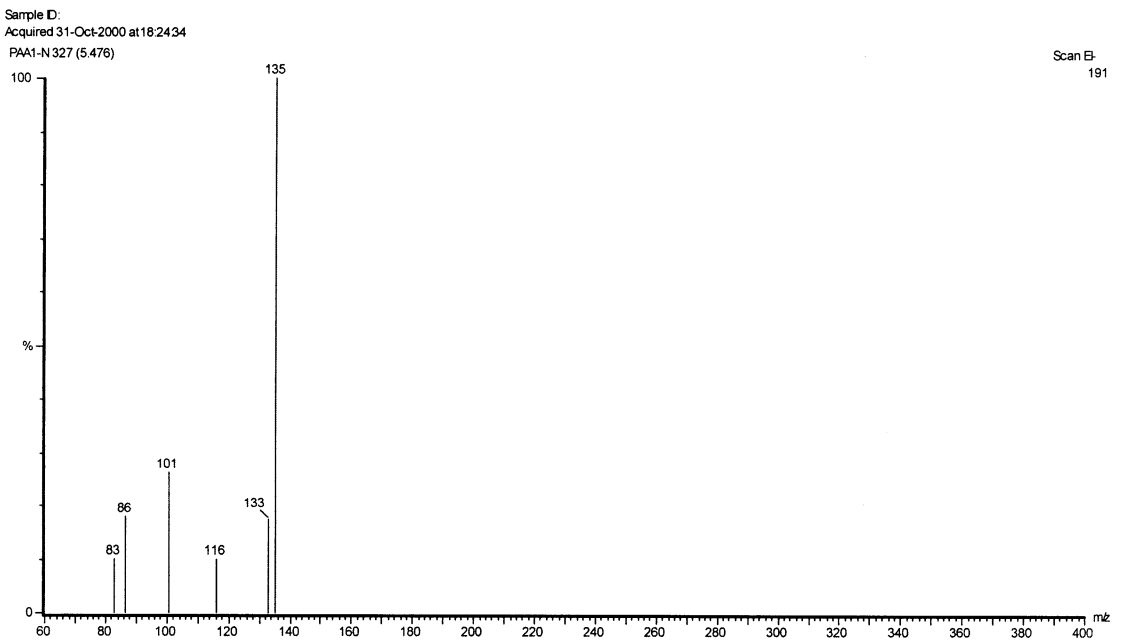
3.4. Optimal conditions

The problem of reproducible enzyme activity is important in the design of reactors employing isolated enzyme. The enzymatic activity is very sensitive to the reaction media. It is known that spiked samples may not always represent the extractability of 'real-world' materials [36]. Therefore, we restored to real sample media at several conditions about introduced optimal conditions of pH, temperature and concentration of Pen G [3–5]

3.4.1. pH conditions

The results at pH values 6, 8 and 9, used for the enzymatic hydrolysis of Pen G, are shown in Fig. 5(a).

An increase of pH, causes the increase of enzyme activity but decreases the half-life of the enzyme. At pH ≥ 9, the probability of decomposition of *E-coli* increases and a by-product appears. However, the results (Fig. 5a) show that a pH value of 8 gives the best result.



(c)

Fig. 3. (*Continued*)

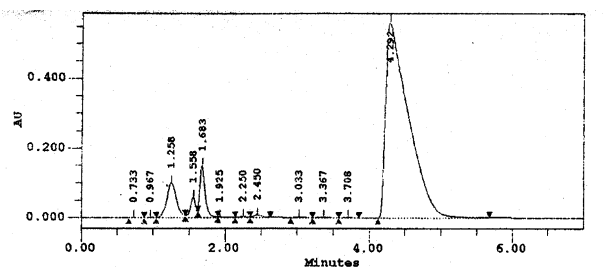
Table 2
2^3 factorial design of pH, temperature and concentration of Pen G enzymatic reaction

Run no.	C (w/v%)	pH	T	Pys-NIMS response	Main effect	Interactive effect
1	6.5(−)	6(−)	18(−)	77.5		
2	12(+)	6(−)	18(−)	27.9		
3	6.5(−)	9(+)	18(−)	77.8		
4	12(+)	9(+)	18(−)	95.6		
5	6.5(−)	6(−)	35(+)	87		
6	12(+)	6(−)	35(+)	64.5		
7	6.5(−)	9(+)	35(+)	117.6		
8	12(+)	9(+)	35(+)	123.1		
	*				39	
		*			28	
			*		−12	
	*	*				23.4
	*		*			39.3
		*	*			−12.2

Fig. 5(b) shows the effect of the type of buffer on the reaction rate. The results show that the buffer type can not significantly effect the reaction rate but HPLC results show that a reaction without a buffering agent, increases benzylpenicilloic acid as a by-product.

3.4.2. Temperature condition

The influence of temperature on the enzymatic hydrolysis of Pen G was evaluated (Fig. 5c). The effect of temperature on the reaction rate can be due to stability of the enzyme acylase and reaction compounds and formation of by-products. Three



Peak Results

#	SampleName	Retention Time (min)	% Area	Area (uV*sec)
1	T=3/1	0.733	0.01	1236
2	T=3/1	0.967	0.01	1228
3	T=3/1	1.258	6.52	931902
4	T=3/1	1.558	2.01	286923
5	T=3/1	1.683	5.38	768689
6	T=3/1	1.925	0.09	12670
7	T=3/1	2.250	0.10	14438
8	T=3/1	2.450	0.31	43781
9	T=3/1	3.033	0.04	5508
10	T=3/1	3.367	0.04	6014
11	T=3/1	3.708	0.02	3574
12	T=3/1	4.292	85.48	12221510

Fig. 4. Chromatogram of enzymatic hydrolysis of Pen G at pH ≥ 9, 6-APA, benzylpenicilloic acid, PAA and Pen G at 1.258, 1.558, 1.683 and 4.292 min, respectively.

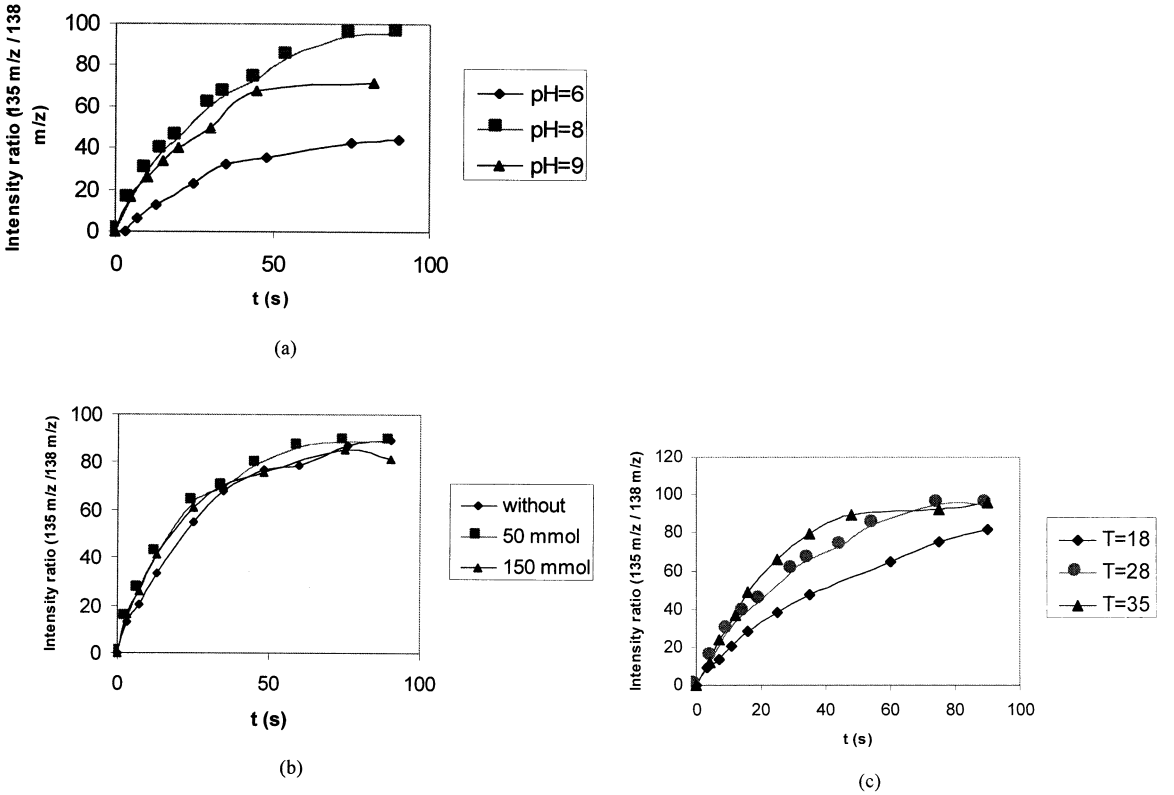


Fig. 5. The effect of (a) pH, (b) buffer type and (c) temperature on the enzymatic hydrolysis of Pen G.

temperatures (18, 28 and 35 °C) were evaluated. Fig. 4(c) shows that the reaction rate at 35 °C is high, but the half-life of enzyme decreases rapidly. Thus 28 °C was selected as the optimized condition of this reaction.

3.4.3. Concentration of Pen G

The enzymatic reaction can be inhibited by the substrate and/or product concentration. In this reaction, PAA is a competitive inhibitor and 6-APA is a noncompetitive agent [37]. Fig. 6 shows that a 12% w/v concentration of Pen G is a suitable condition for this reaction.

Therefore, the optimal conditions of pH, temperature and concentration of Pen G are 8, 28 °C and 12% w/v, respectively.

4. Conclusion

We have shown here that Pyr-NIMS with internal standard can be used for accurate determination of Pen G, 6-APA and PAA and to identify them correctly in enzymatic hydrolysis of Pen G. Pyr-NIMS provides a rapid/specific way of detecting these compounds without lengthy sample preparation steps. Results from this study represent the main and interactive effects of pH, temperature and concentration (C) of Pen G in this reaction.

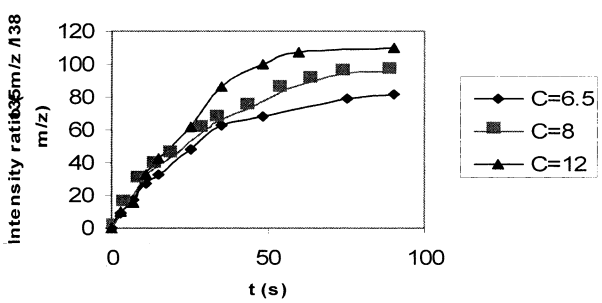


Fig. 6. The influence of concentration of Pen G on the enzymatic hydrolysis of Pen G.

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