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Short communication

Determination of tyramine in cheese by LC–UV

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Abstract

An isocratic reversed-phase liquid chromatographic assay for tyramine has been developed. The method is based on the reaction of tyramine with 4-chloro-7-nitrobenzofurazan and measurement of the absorbance at 458 nm after chromatographic separation on a C-18 column. Optimum reaction conditions were investigated. A linear relationship was found between absorbance and concentration over the range 25–300 ng per 10 μl of tyramine. The method was applied to the determination of tyramine in cheese. The cheese sample was homogenized with 5% (w/v) $HClO_4$ extracted with ethyl acetate–acetone (2:1) and chromatographed on high performance liquid chromatography (HPLC) after derivatization reaction with NBD-Cl. The determination limit was 25 μg/g cheese. The mean recovery of tyramine from cheese was 98.0%.

Keywords: Tyramine; HPLC; Spectrophotometric detection; NBD-Cl; Cheese

1. Introduction

Tyramine, 4-(2-aminoethyl)phenol, the biogenic amine derivative of tyrosine is an active pressor amine, which occurs naturally in many foods and especially in fermented food products. Tyramine is formed from decarboxilation of milk amino acids by metabolic activity of microorganisms during the cheese producing processes. Tyramine usually does not cause any hazard to individuals unless large amounts are digested or the normal catabolism ways are inhibited. Ingested tyramine is largely inactivated by metabolism to *p*-hydroxyphenylacetic acid catalyzed by monoamine oxides (MAO) enzymes. Patient taking MAO inhibitors such as antidepressants or antitubercular drugs can suffer a serious hypertansive reaction if they eat tyramine rich foods. Therefore, it is important to determine the tyramine amount in the foods. Significant amounts of tyramine occur in cheese and its amount varies according to the type of cheese or starter culture, temperature, pH, salt concentration etc. The hypertensive reaction is well known and named as 'cheese effect' or 'cheese reaction' since tyramine was first determined in cheese.

Several methods have been developed for the quantitative determination of tyramine in cheese and other foods. These are generally based on high performance liquid chromatographic (HPLC) ana-

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lysis after the extraction step. Following the chromatographic separation tyramine has been detected by using either UV-detector [1–3] or fluorimetric detector after derivatization with fluorigenic reagents such as dansyl chloride [4,5], *o*-phtalaldehyde [2,6] fluorescamine [7,8]. An HPLC method with amperometric detection system has been also used for the same purpose [9]. Tyramine has been analyzed by spectrofluorimetric method [10,11] as well.

In this study a new HPLC method was developed for the determination of tyramine in cheese. Tyramine was derivatized with 4-chloro-7-nitobenzofurazan (NBD-Cl) reagent, widely used for the estimation of amines [12,13], after the homogenization and extraction steps. NBD-derivative of tyramine was chromatographed on HPLC and detected using absorbance detector. The results were checked using an other HPLC method [14] reported by Moret et al.

2. Experimental

2.1. Apparatus

The HPLC device (Thermo Seperation Products, Texas, USA) consisted of P 4000 solvent delivery system equipped with a Rheodyne injection valve with 20 μl loop, UV 3000 detector and a SN-4000 automation system software was used. The column was Phenomenex Luna (150 × 4.6 mm) packed with RP-18, 5 μ, with a guard column fitted with the same material. Chromatograms were evaluated using isocratic system at ambient temperature. The mobile phase was MeOH–H_2O (70:30) at 1 ml/min flow rate.

Same chromatographic system with gradient elution was used for the control of the results, whereas mobile phase was CH_3CN–0.01 M phosphate buffer (pH 7)–water and detection at 254 nm [14].

Homogenization of the cheese sample with perchloric acid solution was carried out by Ika-Werk, Ultra-Turrax homogenizator. Centrifuge Janetzki T.32 B was used for the separation of liquid phase, after the homogenization process.

2.2. Materials and chemicals

Tyramine, HPLC grade of methanol, acetonitrile and other solvents and chemicals were obtained from Merck (Darmstadt, Germany).

2.3. Solutions

Standard tyramine solutions were prepared from the stock solution, which is prepared dissolving accurately weighed tyramine hydrochloride that is equivalent to 20 mg tyramine in 100 ml of water, by appropriate dilutions with water. NBD-Cl solution was freshly prepared in methanol at 13 and 18 mg/ml concentrations for calibration and analysis of the cheese samples, respectively. Buffer solutions were prepared adjusting desired pH of the solution of 0.6189 g boric acid and 0.7456 g potassium chloride in 50 ml of water with 0.1 M NaOH solution and then volume of the mixture was made up to 100 ml with water. About 5% (w/v) of $HClO_4$ solution was prepared from concentrated perchloric acid by dilution with water.

2.4. Calibration

Aliquots of 25–300 μl of standard solution at 0.3 mg/ml concentration were mixed with 0.1 ml of buffer solution at pH 9.5 and 0.2 ml of NBD-Cl solution at 13 mg/ml concentration in a 5 ml of glass and stoppered tubes. After adding necessary amount of water to complete the volume to 0.3 ml, the mixture was heated at 60 °C for 40 min in a block heater. The mixture was extracted with 3 ml of ethyl acetate on a vortex mixer after cooling and acidifying with 0.1 ml of 0.1 M HCl. One milliliter of ethyl acetate phase was transferred into another tube and evaporated at 40 °C under nitrogen. The residue was dissolved in 1 ml of mobile phase, filtered and 10 μl of this solution was injected to C-18 column.

The chromatogram was achieved using methanol–water (75:25) mobile phase and the peak areas of the NBD-tyramine were determined at 458 nm. Calibration graph was prepared by plotting the concentrations against the peak areas of the derivative.

2.5. Sample preparation

Three grams of cheese sample pressed in a glass mortar, was accurately weighed in a 25 ml of a glass beaker and homogenized with 10 ml of $HClO_4$ solution for 2 min. The mixture was centrifuged at $3000 \times g$ for 10 min and 1 ml of supernatant was transferred into another tube. The solution neutralized with 3% ammonia solution, was saturated with NaCl and extracted with 2 ml of ethyl acetate–acetone (2:1) solvent system on a vortex mixer for 2 min. Then the mixture was briefly centrifuged, organic phase was separated and dried with anhydrous Na_2SO_4 and 1 ml of the extract was evaporated to dryness under nitrogen at 40 °C.

2.6. Assay procedure

The residue obtained from the extraction process was treated with 0.1 ml of borate buffer and 0.2 ml of NBD-Cl solution (18 mg/ml) then reacted as described above at Section 2.4. The amount of tyramine in cheese samples was calculated using the regression equation of the calibration graph.

2.7. Linearity and recovery

The linearity between tyramine concentration and peak areas was determined with the aqueous solutions containing 25–300 ng tyramine in 10 μl. Tyramine was added at three different levels 33.3, 66.7 and 100 μg to 1 g cheese and the recovery was estimated by analyzing these samples.

2.8. Reproducibility and reliability

Reproducibility was calculated from the results of recovery studies. Reliability of developed method was checked by analyzing tyramine content of the same cheese sample by both developed and comparison HPLC methods. In the latter method the residue of cheese extract was dissolved in 1 ml of acetone, derivatized with dansyl chloride reagent and then chromatographed under the conditions mentioned in Section 2[14]. The results obtained by the two methods were compared statistically in terms of mean and R.S.D. values.

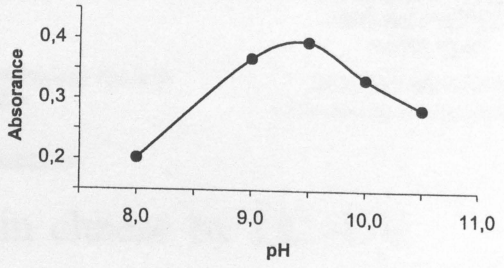

Fig. 1. Effect of pH on the derivatization reaction between tyramine and NBD-Cl.

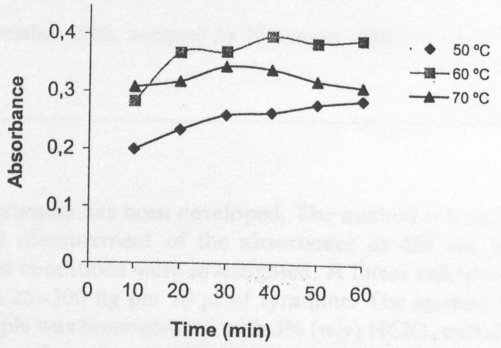

Fig. 2. Effect of temperature and time on the derivatization reaction between tyramine and NBD-Cl.

3. Results and discussion

The reaction between tyramine and NBD-Cl in alkaline medium produced a yellow NBD-tyramine derivative with absorption maximum at 458 nm. Experimental parameters effecting the reaction such as pH, amount of the reagent, temperature and heating time were optimized. For this purpose the reaction was carried out using borate buffer at different pH values between 8.0 and 9.5. The area of the NBD-tyramine peak was measured after acidifying, extraction and chromatography steps as described above. It was determined that the maximum peak area was obtained when the pH of the reaction medium was 9.5 (Fig. 1). The reaction was examined at 50, 60 and 70 °C and it was stopped at 10 min intervals for 60 min. Optimum reaction temperature and time were established as 60 °C and 40 min, respectively (Fig. 2). Necessary reagent amount was determined by varying the mole ratio of NBD-Cl to

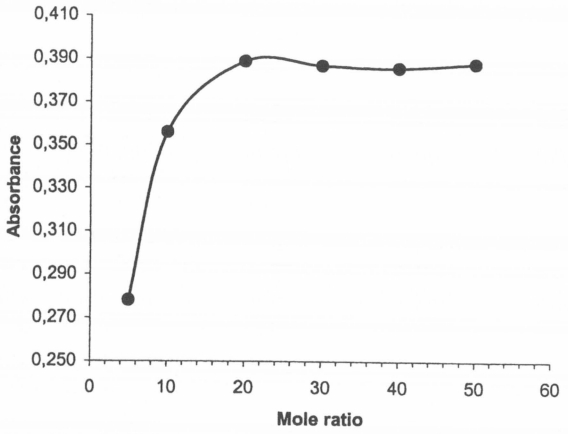

Fig. 3. Effect of the reagent amount on the derivatization reaction between tyramine and NBD-Cl.

tyramine from 10 to 100 and it was found that a 20-fold molar excess was sufficient (Fig. 3).

A linear correlation was obtained between absorbance (A) and tyramine concentration (C) over the range 25–300 ng per 10 μl in the final solution when the reaction was carried out under the optimum conditions described above. The regression equation is $A = 3.47C + 7.71$ ($r = 0.9997$). R.S.D. values for the slope and intercept were 0.55 and 4.46%, respectively.

Tyramine is stable both in aqueous solutions for at least 1 week and during the analysis. The NBD-derivative of tyramine is also stable in the dark and at 4 °C in ethyl acetate for at least 1 day. The fluorescence intensity of the solution does not change on exposure to UV light for 10 min.

The method developed was applied to the assay of tyramine in cheese samples. For the extraction of tyramine from cheese, some examinations were carried out considering the studies reported in the literature. For this purpose aqueous solutions of $HClO_4$, H_3PO_4 and trichloroacetic acid at 5% (w/v) concentration and organic solvents as methanol, ethyl acetate and diethyl ether were tried. It was established that a second extraction step was necessary since many compounds in cheese inter-

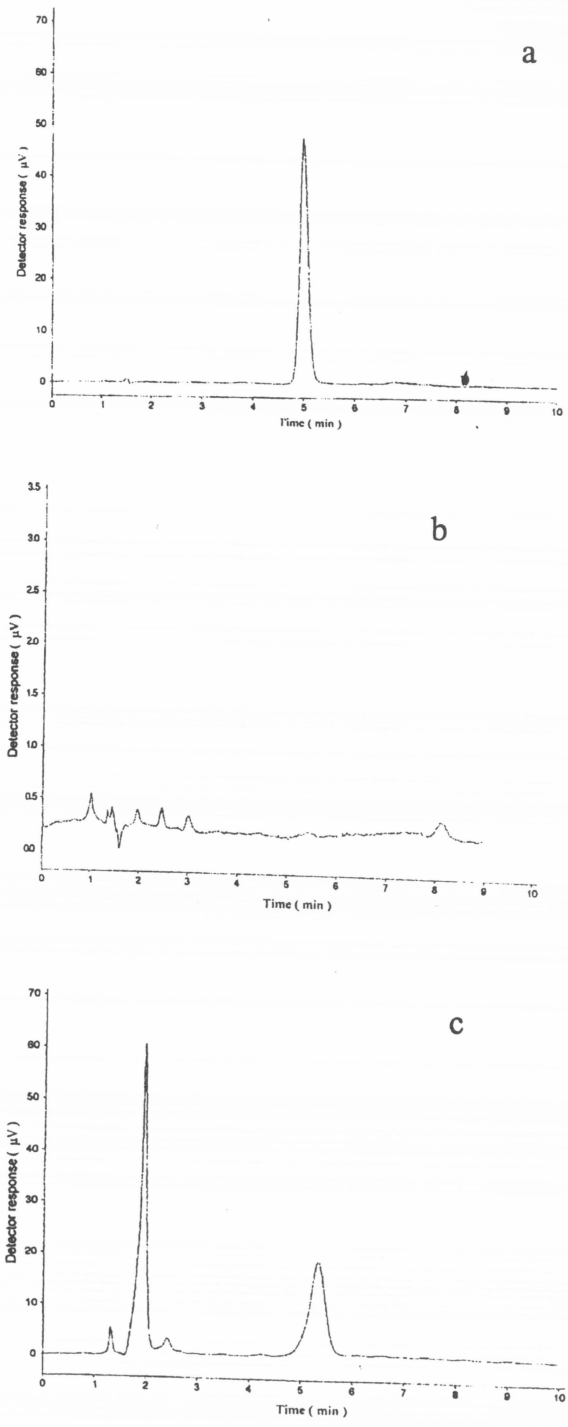

Fig. 4

Fig. 4. HPLC chromatograms of tyramine (a) and blank (b) in aqueous solution and cheese extract (c) after the derivatization reaction.

Table 1
Results of tyramine from spiked and unspiked cheese samples ($n = 5$)

Statistical value	Tyramine amount in sample (μg/g)	Tyramine amount in spiked samples (μg/g)		
		33.3	66.7	100
$\bar{X}$	188.7	221.1	247.4	281.8
S.D.	5.47	6.37	6.01	6.81
R.S.D. (%)	2.90	2.88	2.43	2.42
$t \times$ S.D./$\sqrt{n}$	6.8	7.9	7.5	8.5
Confidence limits	181.9–195.5	213.2–229.0	239.9–254.9	273.3–290.3
Recovery (%)		99.6	96.9	97.6

Table 2
The results of tyramine obtained by developed and comparison HPLC methods ($n = 5$)

	Developed method		Comparison method
Mean	188.7[a]		183.8[a]
S.D.	5.47		9.65
R.S.D.	2.9		5.25
		$t = 0.98$	
		$F = 3.11$	

$t_{teor} = 2.31$; $P = 0.05$. $F_{teor} = 6.39$; $P = 0.05$.
[a] μg tyramine per g cheese.

fere when acidic or organic extract was chromatographed after derivatization reaction with NBD-Cl. Solid phase extraction using C18 cartridges or liquid phase extraction using organic solvent systems were tried. After these examinations, most effective result with high recovery was obtained when the cheese sample was homogenized with $HClO_4$ at a concentration of 5% (w/v) and then extracted with ethyl acetate–acetone (2:1) solvent system after centrifugation and neutralization with ammonia.

Acidic homogenized mixture must be centrifuged using a device with high speed otherwise it is not possible to obtain reproducible results. The sample preparation method is Hurst et al. method [2], used for chocolate samples, except defatting with petroleum ether and holding overnight at 4 °C. An excess of the reagent was used for derivatization reaction with the substances in cheese with a similar structure to that of tyramine.

The chromatograms of tyramine and blank aqueous solutions and cheese extract obtained after the derivatization reaction were given in Fig. 4. Retention time of NBD-tyramine derivative and run time is 6 and 10 min, respectively. NBD-Cl, by products and endogenous substances in cheese do not interfere with the analysis.

The mean recoveries of tyramine from cheese sample is in the range of 96.9–99.6% at three different concentrations and the R.S.D. values are below 2.9% ($n = 5$) (Table 1). The limit of quantitation (LOQ) and limit of detection (LOD) values for tyramine are 25 and 8 μg/g, respectively.

Reliability of proposed method was checked by analyzing tyramine content of the same cheese sample both by developed method and HPLC method reported by Moret et al. [14]. There is not a significant difference between the results obtained both by proposed and direct spectrophotometric methods in terms of accuracy and reproducibility. The results of t- and F-tests were given in Table 2.

The assay of tyramine in cheese was not affected by slight variations either in the pH value of buffer solution or temperature during derivatization process. Slight variations in retention time were observed when the mobile phases prepared on different days by different analysts were used.

4. Conclusion

In this study, it was preferred to improve an HPLC method using spectrophotometric detector for the assay of tyramine in cheese samples although NBD-tyramine derivative has an intensive fluorescent like other NBD-amine derivatives.

Since the absorbance detectors are more widely used than the fluorimetric ones in HPLC analysis and on the other hand, the amount of tyramine in cheese is not too low. Determination limit of the proposed method (25 μg/g) is sufficient for the purpose and almost the same with that of the comparison method (20–100 μg/g) [14], although the sensitivities of the HPLC methods with fluorimetric [2,4–6] or amperometric [9] detectors are very high. The determination limits of the last methods are 1–5 and 0.3 μg/g, respectively. The application of developed method can be extended to the determination of tyramine in biological samples as plasma or urine, by using a fluorimetric detector, because in that case the sensitivity increases about 100-times.

Extraction percent of tyramine from cheese using proposed method is rather high. On no interferences from the compounds present in cheese were observed, that is a very clear chromatogram has been obtained after applied extraction and derivatization steps. R.S.D. values of the method, less than 2.90, indicate good precision. There is no significant difference between the results obtained from proposed and comparison HPLC methods with respect of mean values and standard deviations.

In fact, developed HPLC method which is based on the derivatization reaction between tyramine and NBD-Cl is a selective, sensitive and reproducible analytical procedure for routine tyramine determination in cheese samples.

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