*Journal of Chromatography*, 356 (1986) 311–319
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 400

# DETERMINATION OF BIOGENIC AMINES IN CHEESE AND SOME OTHER FOOD PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN COMBINATION WITH THERMO-SENSITIZED REACTION DETECTION

H. M. L. J. JOOSTEN* and C. OLIEMAN
*Netherlands Institute for Dairy Research, Kernhemseweg 2, 6718 ZB Ede (The Netherlands)*
(Received December 9th, 1985)

## SUMMARY

A simple high-performance liquid chromatographic (HPLC) analysis is described for biogenic amines in cheese and other food products. The sample clean-up consists of a precipitation–extraction step with trichloroacetic acid, which gives recoveries of amines in cheese in the range of 85–105%. The HPLC analysis is performed by reversed-phase ion-pair chromatography, using a ninhydrin-containing eluent, which eliminates the need for an extra reagent pump for the post-column derivatization. Band broadening is minimized by using a poly(tetrafluoroethylene) knitted tube reactor at 145°C. The detection limit for amines is 2 mg/kg cheese and the method is linear for 0.1–4 μg of amine injected. Examples are given of the analysis of amines in cheese, wine and sauerkraut.

## INTRODUCTION

During the ripening of cheese, proteins are degraded by enzymes, resulting in an increase of the free amino acid content. Decarboxylation of the amino acids by bacterial enzymes gives rise to the formation of biogenic amines[1–4]. While in most cheeses the content of biogenic amines is low, some have rather high levels. The consumption of large quantities of amines brings about symptoms of intoxication such as headache, nausea, hypo- or hypertension, cardiac palpitation and possibly shock[5,6]. The amines which may be found in cheese are tyramine, histamine, putrescine, cadaverine, tryptamine and phenylethylamine[7–11].

In order to prevent the formation of toxic amounts of biogenic amines in cheese, an investigation to reveal the essential parameters of the production of amines, was commenced. Therefore we needed a simple method for the quantitative detection of biogenic amines in cheese, making possible an automated analysis of many samples.

A variety of methods for estimating biogenic amines in foods has been described[12]. Most of these deal with the detection of only one or two amines[13–16]. Many methods have been developed for the determination of histamine in fish, since his-

0021-9673/86/$03.50 

tamine is the causative agent of the food poisoning called "scombroïd fever"[17]. Generally applicable methods for the determination of biogenic amines other than histamine and tyramine are usually based on gas or high-performance liquid chromatography (GLC and HPLC respectively). GLC methods always require a derivatization step to make the amines volatile[18,19]. Derivatization, however, is cumbersome and is difficult to reproduce in the presence of an interfering matrix of compounds in food products like cheese. Pre-column derivatization with, *e.g.*, dansyl chloride, followed by HPLC analysis of the products thus formed, suffers from the same drawbacks.

The separation of underivatized amines by using ion-pair chromatography is a well known HPLC technique. UV detection permits the detection of tyramine and tryptamine at 280 nm, phenylethylamine at 254 nm and histamine at 215 nm. However, 215 nm gives no selectivity and the risk of interfering matrix components cannot be neglected. Putrescine and cadaverine, lacking a suitable chromophore, cannot be detected by UV spectroscopy.

Electrochemical detection of underivatized amines by oxidation of the amino group is a sensitive method but not a very useful one since the oxidation takes place only at pH $> 7$ and this means that the separation has to be carried out in an alkaline mobile phase, which excludes the use of silica-based columns. The use of a reagent pump has been discarded since electrochemical detection is far too sensitive for shifts of the eluent composition due to pulsation of the pumps. Reversed-phase packing materials, based on organic polymers, should enable the analysis of biogenic amines in the suppressed ion mode at pH $> 8$. Preliminary experiments with the PRP-1 column, packed with polystyrene, cross-linked with divinylbenzene, were disappointing. Tryptamine and phenylethylamine showed strongly tailing peaks, which could not be suppressed by manipulating the mobile phase.

Post-column derivatization of amines is a widely used technique. With ninhydrin a coloured product is formed[20], while with *o*-phthalaldehyde a highly fluorescent derivative[21] is obtained. A disadvantage of these methods is the requirement of a second pump to deliver the reagent.

Recently, LePage and Rocha[22] described a simple method for the determination of low-molecular-weight aliphatic amines which involved post-column derivatization with ninhydrin. The simplicity is due to the fact that ninhydrin is already dissolved in the mobile phase: the chemical reaction which yields a blue product does not take place until the eluent is heated in a reaction coil (after passage through the column).

The inherent simplicity of thermo-sensitized reaction detection makes this method highly suitable for routine analysis of biogenic amines, requiring in addition to a normal HPLC apparatus only a heated reaction coil. Therefore we have adapted this method for the determination of biogenic amines in cheese and some other food products.

## EXPERIMENTAL

### *Chemicals*

Tyramine (TA), histamine (HA), putrescine (PTR), cadaverine (CAD) and tryptamine (TPA) were obtained from Fluka (Buchs, Switzerland), phenylethylamine (PHEA) from Merck (Darmstadt, F.R.G.), ninhydrin and hydrindantin from Pierce

(Rockford, IL, U.S.A.) and sodium dodecyl sulphate (SDS) from Bio-Rad Labs. (Richmond, CA, U.S.A.). Other reagents were obtained from various suppliers.

*HPLC equipment*

A Waters M6000A pump (Waters Assoc., Milford, MA, U.S.A.) and a M440 absorbance detector (detection wavelength 546 nm) were used together with a Perkin-Elmer ISS 100 automatic sampler, equipped with a 1-ml sample loop, and a Sigma 15 B data system (Perkin-Elmer, Oak Brook, IL, U.S.A.). The separations were performed on a radial compression cartridge (10 × 0.8 cm I.D.), custom-packed with Nucleosil $C_{18}$, 10 μm (Macherey, Nagel & Co., Düren, F.R.G.), in combination with a Waters RCM module. The guard column (3 × 0.3 cm I.D.), was packed with Corasil $C_{18}$ (Waters). The reaction coil consisted of 10 m of PTFE tubing (0.25 mm I.D., volume 0.49 ml) coiled in the form of a twisted figure eight, according to Engelhardt and Neue[23] and heated in an oil-bath to 145°C with a Colora KS ultrathermostat (Colora, Lorch/Württ, F.R.G.).

*Mobile phase*

Ninhydrin (16 g) and 1.2 g hydrindantin were dissolved in 322 ml dimethyl sulphoxide (DMSO) by sonication for 10 min, followed by the addition of 350 ml of 2.8 *M* sodium acetate buffer (pH 5.00). A 2-g amount of SDS was dissolved in a mixture of 618 ml DMSO and 710 ml water. The two solutions were combined and filtered through a 0.45-μm filter. The mobile phase was stored in a dark bottle and nitrogen was constantly passed through the solution. Under these circumstances it remained stable for at least 1 week. The HPLC system was equilibrated for *ca.* 4 h at 1.0 ml/min. The temperature of the RCM module was kept constant at 29°C by means of a small thermostat. A mixture of DMSO and water (1:1) was used to flush the HPLC system before shut-down.

*Extraction of the amines*

For the analysis of cheese and chocolate, 45 ml of a 0.07 *M* trisodium citrate solution (45°C) were added to 5 g of the ground sample and homogenized with a "stomacher" for 5 min. A portion (3 ml) of this suspension was mixed with 3 ml of 0.6 *M* trichloroacetic acid (TCA) and centrifuged for 10 min at 10 000 *g* and 4°C in a Sorvall centrifuge. The resulting pellet was resuspended in 3 ml of 0.3 *M* TCA and centrifuged. The combined supernatants were filtered through a 0.45-μm Gelman acrodisc and the volume was adjusted to 10 ml with water. Wine (3 ml) was treated with 3 ml of 0.6 *M* TCA. After centrifugation, the supernatant was filtered and injected. Fish and sauerkraut were prepared for analysis by adding 200 ml of water to 200 g of sample and blending in a household mixer for 3 min. To 3 ml of the suspension were added 3 ml of 0.6 *M* TCA. After centrifugation the supernatant was filtered.

*Stock solutions of biogenic amines*

Stock solutions containing about 0.2 mg amine per ml in water were kept refrigerated at −20°C. Tryptamine (TPA) solutions were prepared fresh weekly. The calibration mixture was prepared daily by mixing equal volumes of the stock solutions, diluted 1 to 10 in 0.3 *M* TCA solution.

## RESULTS AND DISCUSSION

### *Optimization of the separation and detection*

In order to make the method of LePage and Rocha[22] suitable for detection of biogenic amines in cheese, the composition of the mobile phase was adjusted. The composition of the ninhydrin-containing eluent, however, is restricted by the low solubility of hydrindantin: a DMSO content of at least 40% is required. This concentration makes necessary the use of a very non-polar ion-pair reagent in order to get sufficient retention of the amines. A second restriction is the pH dependence of the ninhydrin reaction: the pH should lie between 5.0 and 5.5. Other components of the mobile phase have a limited solubility, and finally the high viscosity of the DMSO–water mixture makes flow-rates of more than 1.5 ml/min impossible. Despite all these limitations we were able to find a set of conditions for a useful separation.

Fig. 1 shows an example of the optimization of the mobile phase. Eluents were prepared with different SDS and DMSO concentrations in such a way as to keep the retention time more or less constant. The effect of increasing the DMSO content can be counteracted by an increase of the SDS concentration. At 44.5% DMSO and 0.5

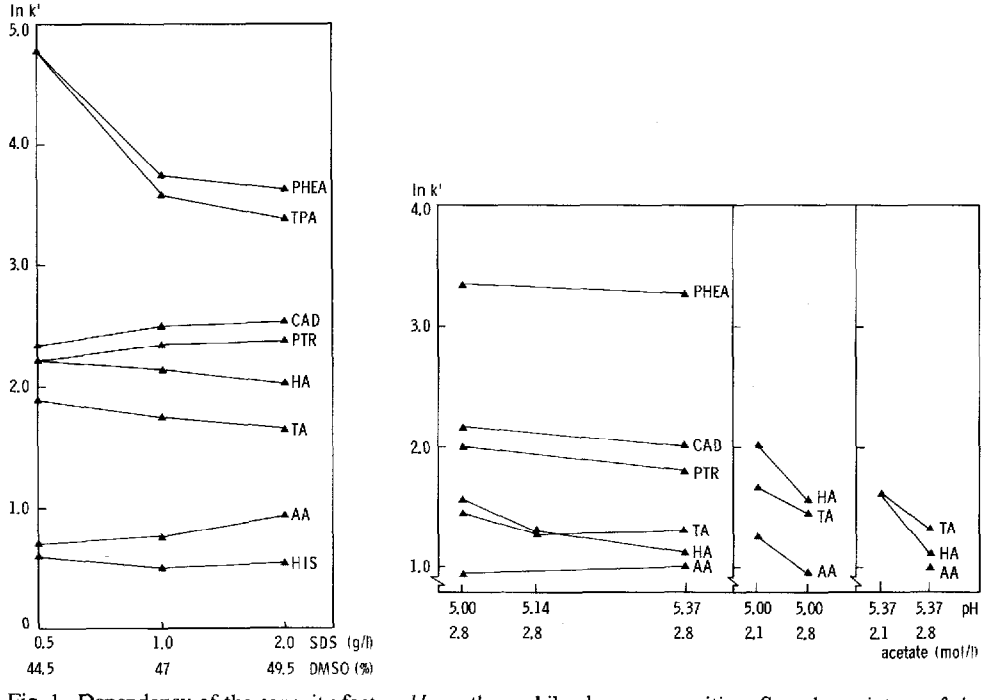



Fig. 1. Dependency of the capacity factor, $k'$, on the mobile phase composition. Samples: mixture of six amines and histidine, 3.3 $\mu$g of each compound per ml and a cheese extract. AA = Last eluted amino acid. Mobile phase: pH = 5.00, 2.8 *M*.

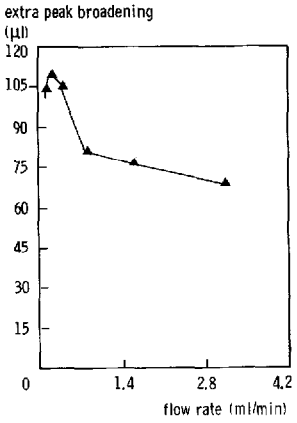
Fig. 2. Dependency of the capacity factor on the pH and molarity of the acetate buffer. Sample: cheese extract spiked with TA, HA, PTR, CAD and PHEA. Mobile phase: DMSO–water (47:53), containing 1 g of SDS per litre.

g SDS/l, there is insufficient separation of putrescine and histamine and of phenylethylamine and tryptamine. With 49.5% DMSO and 2.0 g SDS/l these separations are much better, but this mixture proved to be less useful for cheese samples because of the presence of very large amounts of free amino acids, which interfered with the accurate measurement of the first amine (tyramine) eluted. The mobile phase containing 47% DMSO and 1 g SDS/l proved to be ideal: good separation of all amines and sufficient resolution between tyramine and the amino acids. During the experiments it became clear that the optimum composition was also dependent on the age of the column: after several months of use the DMSO percentage had to be gradually lowered to 43%. This phenomenon is most probably caused by the gradual hydrolysis of the bonded phase.

Another aspect of optimization is shown in Fig. 2. Eluents having small differences in pH and in the molarity of the sodium acetate buffer were prepared. Within the narrow pH range of 5.0–5.4 the degree of protonation of the amino groups of the amines hardly changes, but histamine is very sensitive to small pH shifts, because of the presence of the imidazole group with a $pK_a$ of about 5.0. The 2.8 *M* buffer with pH 5.37 gave a good separation of the first three amines eluted, but analysis of cheese samples caused problems with the resolution of histamine and the amino acids of the cheese extract. Decreasing the molarity of the acetate buffer to 2.1 *M* at pH 5.00 resulted in retention times of more than 2 h for phenylethylamine, which is not a practical proposition, while the resolution between the amino acids and tyramine decreased. Optimum results were obtained with the 2.8 *M* buffer at pH 5.00.

The use of methylcellosolve, which is more toxic than DMSO, gave more problems in keeping the hydrindantin dissolved, while the separation of the amines was less satisfactory than with DMSO.

A reduction of the analysis time by using gradient elution was not possible, since an unacceptable baseline drift was observed. This might be caused by the slow



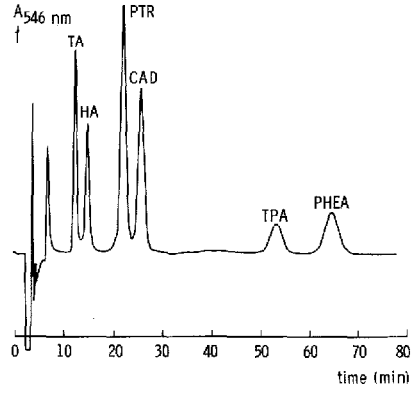




Fig. 3. Extra peak broadening of the knitted tube reactor at different flow-rates of water. A 5 µl volume of acetone was injected into a system with and without the knitted tube reactor.

Fig. 4. Separation of a mixture of biogenic amines, in combination with thermo-sensitized reaction detection with ninhydrin. Detection wavelength: 546 nm. Injected amount: 0.6 µg of each amine. For the column and conditions, see Experimental.

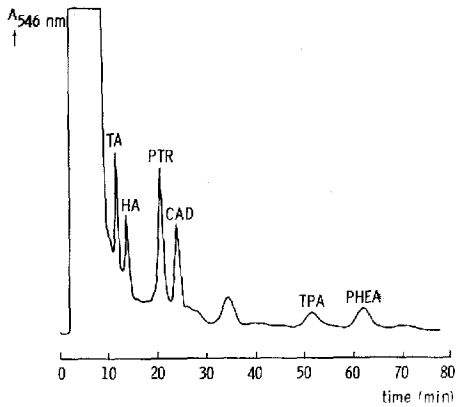



Fig. 5. Chromatogram of a cheese extract, spiked with the amine mixture (each 50 mg per kg cheese). Amino acids are eluted during the first 10 min.

equilibration of SDS with the stationary phase or by desorption of ninhydrin or hydrindantin during the gradient elution.

The band broadening caused by the post-column reaction coil as a function of the flow-rate is shown in Fig. 3 for water. At a flow-rate of 1.0 ml/min an extra band broadening of only 80 $\mu$l was measured, having no influence on the resolution. The optimum reaction temperature was found to be 145°C.

*Analysis of biogenic amines*

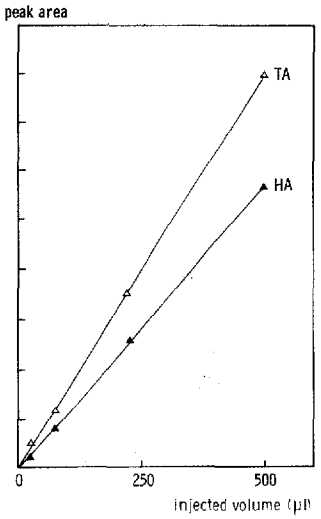
Figs. 4 and 5 show chromatograms of a standard mixture and of a Gouda cheese extract, spiked with a mixture of the amines. An analysis time of 1.5 h is needed for the complete separation of the important amines in cheese. Despite the long retention times of tryptamine and phenylethylamine, these compounds show good peak shapes and they have response factors comparable to those of the faster eluting amines. The rate of the reaction of ninhydrin with amines at 30°C is very low and does not interfere with the analysis. The amino acids are eluted during the first 10 min. Since these are present in very high amounts in cheese, massive peaks are observed on the chromatogram. By lowering the amount of extract injected it is also

TABLE I

RECOVERY OF AMINES BY THE TCA PRECIPITATION METHOD

$n$ = 2. The cheese used contained 5 mg histamine and 5 mg putrescine per kg.

| *Added amount (mg/kg cheese)* | *Recovery (%)* | | | | | |
|---|---|---|---|---|---|---|
| | *TA* | *HA* | *PTR* | *CAD* | *TPA* | *PHEA* |
| 10 | 140 | 95 | 73 | 100 | 140 | 140 |
| 50 | 102 | 86 | 86 | 94 | 92 | 94 |
| 100 | 99 | 93 | 98 | 95 | 100 | 95 |
| 500 | 93 | 96 | 99 | 97 | 103 | 102 |
| 1000 | 92 | 94 | 97 | 95 | 102 | 102 |
| 2000 | 93 | 94 | 94 | 94 | 97 | 102 |



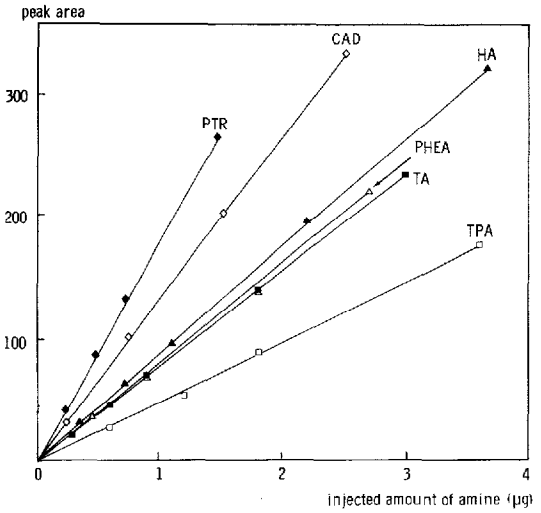




Fig. 6. Peak area as a function of the injected volume (25–500 µl). Sample: mixture of tyramine and histamine (each 3.3 µg/ml). For the column and conditions, see Experimental.

Fig. 7. Linear regression of peak area and the amount of injected amine. Samples of 200 µl were injected, containing 0–4 µg of each amine. Residual standard deviations: TA, 0.3; HA, 0.3; PTR, 2.2; CAD, 0.6; TPA, 5.4; PHEA, 22.6; statistically, the intercepts did not differ from zero.

possible to get an impression of the total amino acid formation in cheese. Histidine (HIS) can be detected selectively by decreasing the content of DMSO to 40% and lowering the molarity of the buffer to 0.7 *M*.

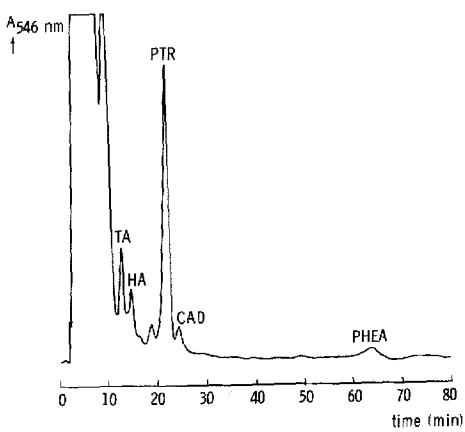
The recovery of amines was studied by homogenizing a three month-old Gouda type cheese with a low amine content ($\leqslant$10 mg/kg). Portions of the suspension

TABLE II

REPEATABILITY

Two cheese samples were fortified with 50 and 500 mg of each amine per kg cheese, respectively. Extracts of these were each injected four times and peak areas were determined. The repeatability of the analysis is expressed as the relative standard deviation (R.S.D.).

| *Amine concn. (mg/kg cheese)* | *Peak area* | | *R.S.D. (%)* | *n* |
|---|---|---|---|---|
| | *Mean* | *Range* | | |
| TA 50 | 25.9 | 25.59–26.27 | 1.21 | 4 |
| 500 | 310.4 | 304.6–315.9 | 1.58 | 4 |
| HA 50 | 21.4 | 21.03–21.65 | 1.47 | 3 |
| 500 | 298.8 | 292.2–308.0 | 2.46 | 4 |
| PTR 50 | 63.3 | 62.16–64.35 | 1.90 | 4 |
| 500 | 627.9 | 617.1–641.2 | 1.92 | 4 |
| CAD 50 | 58.1 | 56.73–59.49 | 3.36 | 2 |
| 500 | 465.6 | 457.1–475.9 | 2.13 | 4 |
| TPA 50 | 14.0 | 12.24–15.15 | 9.94 | 4 |
| 500 | 136.5 | 133.8–137.5 | 1.31 | 4 |



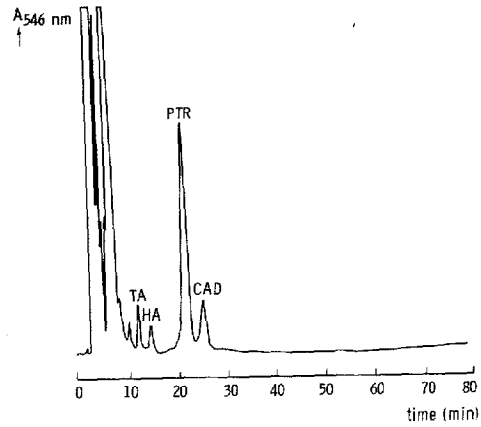




Fig. 8. Chromatogram of a wine extract. This sample contained tyramine (2.7 mg/l), histamine (2.0 mg/l), putrescine (6.9 mg/l) and also traces of cadaverine and phenylethylamine.

Fig. 9. Chromatogram of a sauerkraut extract, containing a large amount of putrescine (210 mg/kg). Tyramine, histamine and cadaverine were also detectable (69, 58 and 79 mg/kg respectively).

were spiked with amines at several concentrations. The results are summarized in Table I. The recoveries of the amines varied between 85 and 105%. Only at the lowest level (10 mg/kg added) was this range larger.

Attempts to make sample preparation easier by reducing the procedure to only one TCA precipitation failed, since recovery rates were about 10% lower. Neither did the addition of 20–40% DMSO to the TCA solution raise the efficiency of the first extraction.

The detection limit for biogenic amines in cheese is 2 mg/kg for each of the amines, which is sufficiently low for toxicological investigations. This low limit is also due to the large sample volume of 200 $\mu$l, which could be injected without any change in peak size and shape (Fig. 6). The amines are concentrated at the top of the column probably because they are dissolved in a weaker eluent (0.3 *M* TCA) than the mobile phase. They will not be eluted as long as the surrounding solution does not contain DMSO.

To investigate the repeatability, two Gouda cheese samples, one with a low and one with a high amine content (50 and 500 mg/kg, respectively), were each analysed four times. The results are summarized in Table II. The sample with the low amine content gave a relative standard deviation (R.S.D.)[24] of less than 10%. The sample with the high amine content showed a maximum R.S.D. of 2.5%.

Assuming a linear relationship between the amount of amine injected and its peak area, the residual standard deviation (Syx) was determined. Constant volumes of different diluted standard solutions were injected and the peak areas thus obtained were subjected to linear regression (Fig. 7).

This method was also tested on several other foodstuffs which are known to contain biogenic amines: fish (tuna), wine, sauerkraut and chocolate. Examples are shown in Figs. 8 and 9 for wine and sauerkraut respectively. The detection limit of the amines in sauerkraut is 0.8 mg/kg and in wine 0.3 mg/kg.

## CONCLUSION

The described HPLC system permits the determination of biogenic amines in cheese and other foods with good sensitivity and specificity. By means of an automatic injector, this method is very useful for routine analysis.

## ACKNOWLEDGEMENT

This work was supported by the Stichting J. Mesdagfonds, instituted by Kaascontrolestation Friesland, Leeuwarden, The Netherlands.

## REFERENCES

1 S. L. Rice, R. R. Eitenmiller and P. E. Koehler, *J. Milk Food Technol.*, 39 (1976) 353.
2 A. Askar, *Ernährungs-Umschau*, 29 (1982) 143.
3 D. Beutling, I. Janz and G. Scheibner, *Mh.Vet.-Med.*, 39 (1984) 231.
4 S. T. Edwards and W. E. Sandine, *J. Dairy Sci.*, 64 (1981) 2431.
5 F. Franzen and K. Eysell, *Biologically Active Amines Found in Man*, Pergamon, New York, 1969.
6 W. Lovenberg, in *Toxicants Occurring Naturally in Foods*, Natl. Acad. Sci., Washington, DC, 1973, p. 170.
7 U. Pechanek, H. Woidich, W. Pfannhauser and G. Blaicher, *Ernährung/Nutrition*, 4 (1980) 58.
8 P. Koehler and R. Eitenmiller, *J. Food Sci.*, 43 (1978) 1245.
9 G. J. Silverman and F. V. Kosikowski, *J. Dairy Sci.*, 39 (1956) 1134.
10 M. Saxby, J. Chaytor and R. Reid, *Food Chem.*, 6 (1981) 281.
11 S. Ramantanis, *Arch. Lebensmittelhyg.*, 35 (1984) 75.
12 N. Seiler, *J. Chromatogr.*, 143 (1977) 221.
13 G. Suhren, W. Heeschen and A. Tolle, *Milchwissenschaft*, 37 (1982) 143.
14 M. Voigt, R. Eitenmiller, P. Koehler and M. Hamdy, *J. Milk Food Technol.*, 37 (1974) 377.
15 J. Chaytor, B. Crathorne and M. Saxby, *J. Sci. Food Agric.*, 26 (1975) 593.
16 T. Chambers and W. Staruszkiewicz, *J. Assoc. Off. Anal. Chem.*, 61 (1978) 1092.
17 S. Hudson-Arnold and W. Duane-Brown, *Adv. Food Res.*, 24 (1978) 113.
18 S. Wada, M. Takada and C. Koizumi, *Chem. Abstr.*, 98 (1983) 52033f.
19 J. Slemr and K. Beyermann, *J. Chromatogr.*, 283 (1984) 241.
20 S. Moore and W. Stein, *J. Biol. Chem.*, 176 (1948) 367.
21 M. Walters, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 1040.
22 J. LePage and E. Rocha, *Anal. Chem.*, 55 (1983) 1360.
23 H. Engelhardt and U. Neue, *Chromatographia*, 15 (1982) 401.
24 *ISO 5725-1981*, International Organization for Standardization, Geneva, 1981.