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Note

Determination of aromatic biogenic amines and their precursors in cheese by high-performance liquid chromatography

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Cheese, like other fermented foods, may contain biogenic amines as a result of decarboxylation of certain amino acids by micro-organisms[1,2]. Biogenic amines are either psychoactive or vasoactive and may cause problems to some consumers[3]. Because of this, research on the formation of biogenic amines in cheese is of interest. Biogenic amines found in cheese are aromatic (such as histamine, tyramine, phenylethylamine and tryptamine) or aliphatic (such as putrescine and cadaverine). The major factors that govern the formation, concentration and type of amines are probably the availability of amino acids (and hence proteolysis of cheese) and the presence of certain bacteria able to decarboxylate amino acids. Even so, in the presence of bacteria with decarboxylase activity and with sufficient proteolysis, biogenic amines are not always formed in cheese, at least not in a high concentration. It must be concluded that the mechanism of formation is not yet fully understood.

In order to study the ways of formation of biogenic amines in cheese, we needed a suitable and relatively fast method, not only for the determination of amines but also of their precursors, because the availability of free amino acids may be the limiting factor. Chromatographic methods available for the determination of biogenic amines are high-performance liquid chromatography (HPLC)[4–7], gas–liquid chromatography[8–10] and thin-layer chromatography[11,12]. All workers (except one group[6], who used UV detection at 254 nm) used a derivatization procedure (mostly fluorimetric) to detect amines. Disadvantages are the need for a derivatization step (and hence laborious clean-up of samples), incomplete derivatization and occasionally the formation of side products[13]. Moreover, most methods do not determine all biogenic amines of interest in the same analysis, let alone the precursors of the amines. A notable exception as far as derivatization is concerned is the procedure of Joosten and Olieman[7], who used a post-column derivatization by incorporating ninhydrin in the eluent, letting the reaction take place after the separation simply by heating; however, their method is not suitable for the determination of amino acids.

Therefore, we developed an HPLC method for the determination of aromatic biogenic amines together with their precursors, namely tyramine–tyrosine, histamine–histidine, phenylethylamine–phenylalanine and tryptamine–tryptophan. The components are detected by their natural absorbance in the UV region. On completion of this work, a similar study was published by Chang *et al.*[14]. They

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focused mainly on the effect of several ion-pairing agents, whereas we focused on the effect of organic modifiers, temperature and detection. Our work and that of Chang *et al.*[14] can thus be seen as complementary. In addition, we determined phenylalanine–phenylethylamine, compounds not determined by Chang *et al.*[14].

EXPERIMENTAL

Reagents

Methanol, acetonitrile, potassium dihydrogen phosphate and trichloroacetic acid were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). Sodium heptane-sulphonate monohydrate was obtained from Fluka (Buchs, Switzerland). Amines and amino acids were obtained from Fluka and Merck.

Apparatus

Spectra-Physics HPLC equipment was used, consisting of an SP 8100 pump–oven–injector (fixed loop), an SP 8110 autosampler, an SP 8440 UV–visible variable-wavelength detector and an SP 4200 computing integrator. Stopped-flow scanning is possible under control of a BASIC program (AUTOSCAN, Spectra-Physics) residing in the SP 4200 computer.

Chromatographic conditions

The mobile phase was a mixture of a buffer with an organic modifier (methanol or acetonitrile). The buffer consisted of a 0.01 *M* sodium heptanesulphonate and a 0.01 *M* phosphate (KH_2PO_4) solution adjusted to pH 3 with orthophosphoric acid. The water used was demineralized and filtered through a 0.45-μm filter. The mobile phase was continuously degassed with helium. The flow-rate was 1.5 ml/min.

A CP Spher C_{18} reversed-phase column (250 × 4.6 mm I.D.) (particle size 10 μm) was used with a guard column (75 × 2.1 mm I.D.) packed with pellicular reversed-phase material (Chrompack, Middelburg, The Netherlands). The column was kept at 40°C unless indicated otherwise. The injection volume was 20 μl.

Detection was by UV absorbance at 215 nm, unless indicated otherwise.

Sample preparation

A 10-g amount of ground Gouda cheese was extracted with 25 ml of trichloroacetic acid (5%, w/v) in a Sorvall Omni-mixer. After cooling to 3°C (to crystallize most of the fat), the sample was centrifuged at 3°C, the creamed layer removed and the supernatant filtered. A 5-ml volume of the filtrate was diluted with water to 50 ml and 20 μl of the diluted filtrate was injected onto the column.

RESULTS AND DISCUSSION

Selection of a suitable wavelength

To determine the eight components of interest in one run it was necessary to select a wavelength in the far-UV region, as can be seen from the spectra obtained by stopped-flow scanning (Fig. 1). No difference was found in the UV spectra of the amines and their corresponding amino acids. A wavelength of 215 nm was selected as being optimal for the simultaneous determination of all eight components. To

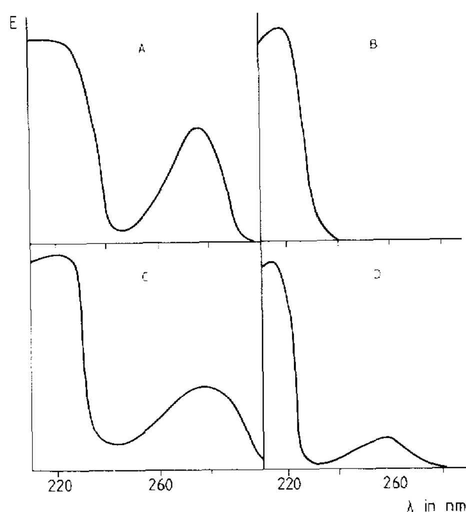


Fig. 1. UV spectra obtained by stopped-flow scanning of (A) tyramine–tyrosine, (B) histamine–histidine, (C) tryptamine–tryptophan and (D) phenylethylamine–phenylalanine.

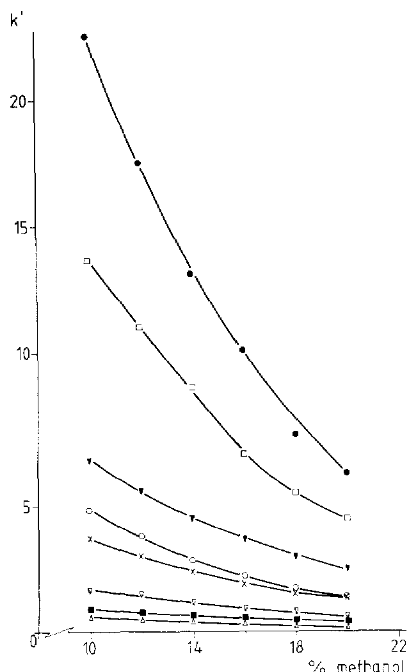
increase the selectivity, however, other wavelengths can be chosen, except for histidine and histamine; this appeared to be useful in some instances to confirm results obtained at 215 nm.

Selection of chromatographic conditions

The separation of ionic or ionizable components (such as amines and amino acids) by reversed-phase HPLC can be achieved by means of ion-pair chromatography[15,16]. Based on the paper by Deelder *et al.*[16], we selected heptanesulphonic acid at a concentration of 0.01 *M*. It was necessary to adjust the pH of the aqueous buffer to 3 so as to prevent dissociation of the carboxyl groups of the amino acids. At higher pH the resolution of the amino acids was insufficient. An organic modifier is needed to achieve a resonable analysis time. Figs. 2 and 3 show the influence of methanol and acetonitrile, respectively, on the capacity factors. No so-called specific effects[17] of these modifiers on the capacity factors were observed; hence, ternary acetonitrile–methanol–buffer mixtures will not improve the separation. Methanol was selected as the organic modifier for further studies. The very high capacity factors of tryptamine and phenylethylamine at low methanol concentrations necessitate the use of a methanol concentration of *ca.* 15%.

Figs. 2 and 3 were obtained at a column temperature of 40°C. This temperature appeared to be optimal; Fig. 4 shows the influence of column temperature on the capacity factor at a methanol concentration of 15%. Clearly, the temperature during an analysis has to be constant, as temperature appears to have a large effect. Comparison of Figs. 2 and 3 with Fig. 4 indicates that the effect of increasing the content of organic modifier is about the same as increasing the column temperature. We did not vary the concentration of the ion-pairing agent; changing this concentration may be another means of optimizing the separation[18].

In conclusion, the factors that govern the retention of amines and amino acids



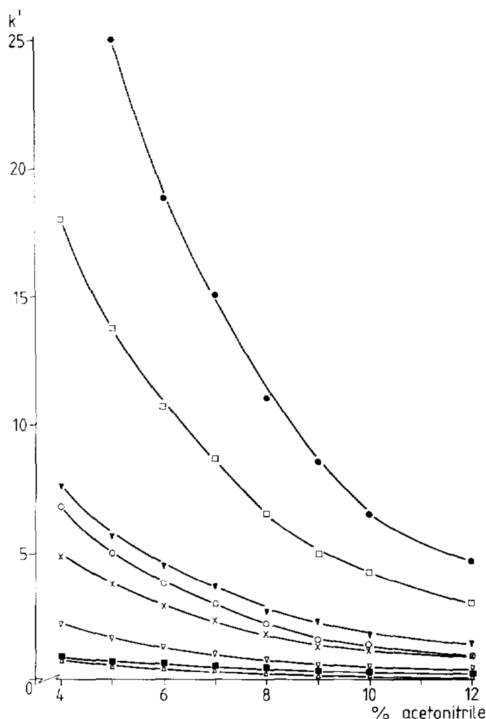


Fig. 2. Capacity factor, k', as a function of the methanol content. (●) Tryptamine, (□) phenylethylamine, (▼) histamine, (○) tryptophan, (×) tyramine, (▽) phenylalanine, (■), histidine, (△) tyrosine.

Fig. 3. Capacity factor, k', as a function of the acetonitrile content. Symbols as in Fig. 2.

are the pH of the buffer, the content and kind of organic modifier and the temperature (at a fixed concentration of the ion-pairing agent). Fig. 5 shows a chromatogram of the four amino acids and the corresponding amines, detected at wavelengths 215 and 260 nm. A linear relationship was obtained between peak area and amount injected over the range 0.1–8 μg for all eight components.

Determination of amino acids and amines in cheese samples

Of four extractants tried [water, an aqueous buffer of pH 4.5, methanol and trichloroacetic acid (TCA)], TCA at a concentration of 5% (w/v) appeared to give the best chromatograms. The recoveries of amines added to a ground cheese with a low amine content at a concentration of 200 mg/kg were (in duplicate) 90, 93% (tyramine), 100, 106% (histamine), 89, 93% (phenylethylamine) and 102, 104% (tryptamine). This is in agreement with literature data on TCA extraction[7,19]. The detection limit for all four biogenic amines is well below 10 mg/kg (concentrations of biogenic amines below this level are not important).

An example of a chromatogram of cheese extracted with TCA is shown in Fig. 6. With some cheeses, interfering compounds appear at the beginning of the chromatogram and may affect the determination of tyrosine. In that event, detection at

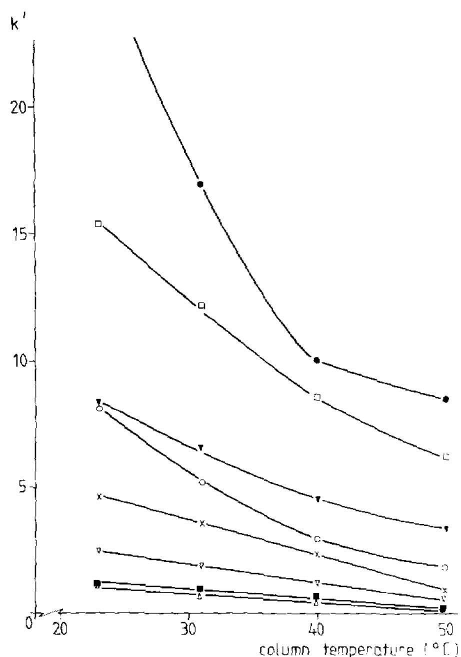


Fig. 4. Capacity factor, k', as a function of column temperature. Methanol concentration, 15%. Symbols as in Fig. 2.

Fig. 5. Chromatograms of a mixture of amine and amino acids. Detection at (a) 215 nm and (b) 260 nm. 1 = Tyrosine; 2 = histidine; 3 = phenylalanine; 4 = tyramine; 5 = tryptophan; 6 = histamine; 7 = phenylethylamine; 8 = tryptamine.

260 nm solves the problem; Fig. 7 shows an example. It should be noted, however, that histidine and histamine cannot be detected at that wavelength.

It seems likely that the interfering compounds result from proteolytic breakdown during the ripening of cheese, but we have not studied this phenomenon in detail. Apart from changing the wavelength, the problem of interfering compounds can also be solved by adjusting the composition of the eluent. However, the analysis may then take a long time (*cf.*, Figs. 2 and 3) and, obviously, this approach is not suitable for routine analysis.

Another problem is that the retention times of the amino acids and amines began to decrease after a considerable run time (*e.g.*, after more than 100 injections). An example of this phenomenon can also be seen in Fig. 7: the resolution between histidine and phenylalanine has decreased. This is probably a consequence of overloading the column with the ion-pairing agent, as the problem disappears after thorough washing with water and subsequently with methanol.

CONCLUSION

This method appears to be suitable fo the rapid determination of aromatic amino acids and amines in cheese. With interfering compounds possibilities exist for circumventing this problem by adjusting the wavelength or the mobile phase com-

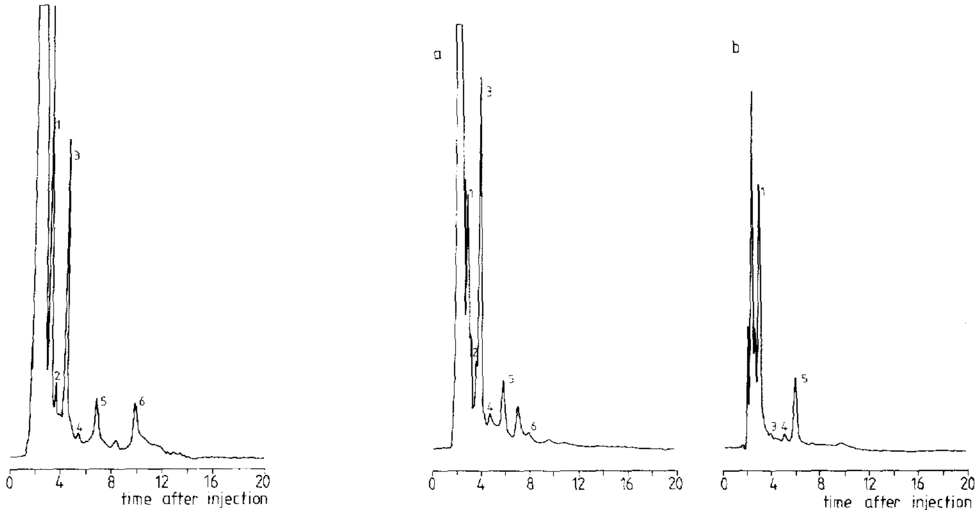


Fig. 6. Chromatogram of a Gouda cheese extract. Peaks as in Fig. 5.

Fig. 7. Chromatogram of a Gouda cheese extract. Detection at (a) 215 nm and (b) 260 nm. Peaks as in Fig. 5.

position. Although we did not verify this, we expect this method to be suitable for the determination of aromatic biogenic amines in other foods. Minor adjustments may be necessary, but means of doing so are indicated in this paper.

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