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Rapid identification of biogenic amine-producing bacterial cultures using isocratic high-performance liquid chromatography

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Abstract

Biogenic amines are extracted from bacterial cultures with an organic solvent using Extrelut packings. The organic extract is subjected to OPA–ethanethiol derivatisation. The derivatives of amines are separated by high-performance liquid chromatography under isocratic conditions.

1. Introduction

Recently much interest has been focused on dairy-related bacterial strains which are able to produce biogenic amines in cheese [1–3]. The presence of such strains is associated with accumulation of histamine, tyramine, tryptamine, isobutylamine, phenethylamine, putrescine and cadaverine. Some of the amines are biologically active, causing psychoactive or vasoactive effects in humans [4,5]. The production of amines is catalysed by specific amino acid decarboxylase enzymes.

A classical procedure for identification of histamine-producing strains includes cultivation of a suspected colony in e.g. *Lactobacilli* MRS-broth[1] supplemented with histidine and testing the grown culture for accumulation of histamine. Non-chromatographic procedures for the assay of histamine include extraction by ion exchange and elution with methanol, followed by derivatisation with *o*-phthaldialdehyde (OPA) [6], or reaction of the broth culture with diamine oxidase and peroxidase to produce a colour [7]. However, these techniques are either un-specific or labour-intensive.

New developments in this area include isocratic reversed-phase HPLC with post-column ninhydrine derivatisation and a separation time of 70 min [8], gradient reversed-phase HPLC using pre-column derivatisation with either OPA [9] or dansyl chloride [10–15] or 9-fluorenylmethyl chloroformate [16], and ion-exchange separation by amino acid analysers [17]. However, dansylation may be associated with both long derivatisation times and the presence of non-reacted dansyl chloride, which in some cases may overload the column and disturb the evaluation of the chromatogram. Furthermore, gradient elution is usually associated with low sample throughput.

The present procedure involves liquid–liquid extraction of a 10-μl bacterial culture sample on an Extrelut packing with an organic solvent. The

[1] According to the formulation of deMan, Rogosa and Sharp.

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eluate is immediately subjected to derivatisation with OPA–ethanethiol, followed by isocratic HPLC. This approach has been extensively used in screening applications to identify either histamine- or tyramine-forming bacterial cultures.

2. Experimental

2.1. Chemical reagents

NaOH, Na_2HPO_4, ascorbic acid, ethanol, ethyl methyl ketone (EMK), isopropanol, acetic acid, methanol gradient grade for HPLC, phthaldialdehyde for fluorescence analysis and acidic aluminium oxide active for column chromatography were from E. Merck. Histamine dihydrochloride, tyramine, tryptamine hydrochloride, isobutylamine, phenethylamine, pentylamine, putrescine dihydrochloride, cadaverine dihydrochloride, ethanethiol and triethylamine for amino acid analysis were from Fluka.

2.2. Materials

10 × 75 mm Pyrex borosilicate glass disposable culture tubes from Corning Inc., 1.5-ml disposable Eppendorf microtubes, 1-ml disposable pipette tips, 12-ml serum vials and Extrelut, a preparation of specially processed wide-pore diatomaceous earth from E. Merck, were used.

2.3. Preparation of solutions

Stock solutions of biogenic amines were prepared at a concentration of 10 mmol/l

Biogenic amine standard mixture was prepared by adding 1 ml of each amine stock solution in a 10-ml volumetric flask and making up with 0.1 mol/l HCl to the mark. The solution was stored in 1-ml volumes at −18°C until use.

Internal standard solution was prepared by pipetting 12 μl pentylamine in 100 ml of 0.1 mol/l HCl. This was stored in the refrigerator.

Sodium ascorbate–phosphate solution pH 12.5 was prepared by dissolving both 50 mmol of ascorbic acid and 50 mmol of disodium hydrogen orthophosphate in 80 ml of 1 mol/l NaOH under both stirring and adjusting with 10 mol/l NaOH to pH 12.5 and by making with water to 100 ml. The solution was stored in tightly sealed 12-ml serum vials, which had been filled up to the top in order to expel the air.

EMK–isopropanol (90:10, v/v) was prepared prior to use. Some batches of EMK produced a shoulder on the histamine peak. This could be prevented by passing 20 ml of EMK through a 4-g bed of acidic aluminium oxide.

OPA reagent was prepared on the day of use by dissolving both 10 mg of OPA and 50 μl of ethanethiol in 1 ml of methanol and by mixing with 9 ml of 0.4 mol/l boric acid adjusted with 10 mol/l NaOH to pH 9.5.

2.4. HPLC apparatus

The HPLC system consisted of a Beckman pump Model 100A, a Rheodyne microsample injector Model 7520 with a 1-μl loop, a Merck-Hitachi fluorescence detector Model F 1000 with a 2-μl flow cell and an Altex Integrator Model C-R1A.

2.5. HPLC column and separation

A 125 × 2 mm I.D. LiChroCart cartridge filled with Superspher 100 RP-18 from E. Merck was used. The mobile phase consisting of 85 ml methanol and 15 ml aqueous 7% (v/v) triethylamine–acetic acid pH 7.5, was delivered at a rate of 0.2 ml/min. Fluorescence detection was performed at 340 nm and 450 nm for excitation and emission, respectively.

2.6. Extrelut packing

A 1-ml pipette tip was filled with 200 mg of Extrelut. Prior to use the packing was conditioned by adding 0.25 ml of sodium ascorbate–phosphate pH 12.5 to the adsorbent. Usable within a few hours.

2.7. Standardisation

This was performed by the internal standardisation method. A 10-μl volume of the biogenic amine standard mixture and a 10-μl volume of the internal standard solution were sequentially applied onto a conditioned Extrelut packing. The amines were eluted with 1.3 ml of EMK–isopropanol (90:10, v/v) or EMK only. The eluate collected in a 10 × 75 mm Pyrex test tube (about 1 ml) was subjected to derivatisation by adding 0.2 ml of the OPA reagent. The tube was capped, shaken on a vortex-mixer for 15 s and allowed to stand for 30 min. One volume of the organic supernatant was diluted with 1 volume of methanol and 2 volumes of water and then injected onto the HPLC column.

2.8. Extraction of biogenic amines from bacterial broth cultures and derivatisation

A 10-μl volume of a bacterial culture and a 10-μl volume of the internal standard solution were sequentially applied onto the Extrelut packing. Steps of elution, derivatisation and separation were identical to those given in the section on standardisation.

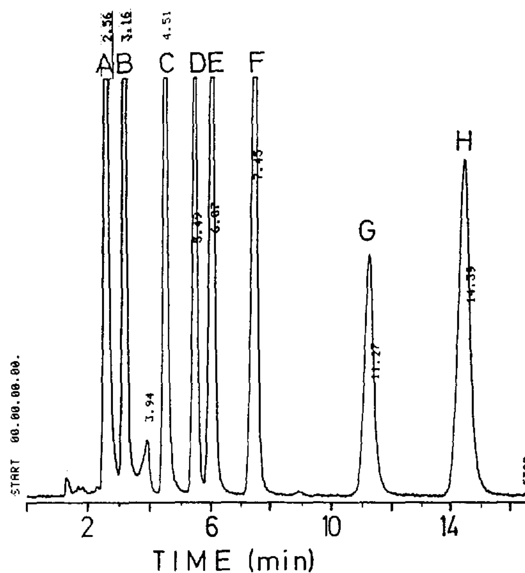

Fig. 1. Separation of biogenic amines using OPA–ethanethiol derivatisation. Derivatisation and injection onto the HPLC column were performed as described in Experimental. Peaks with retention times (min) are as follows: A = histamine, 2.56; B = tyramine, 3.16; C = tryptamine, 4.51; D = isobutylamine, 5.49; E = phenethylamine, 6.07; F = pentylamine, 7.45; G = putrescine, 11.27; and H = cadaverine, 14.39. Separation conditions: mobile phase flow, 0.2 ml/min; injection volume, 1 μl; attenuation, 16 mV/full scale.

3. Results and discussion

Fig. 1 shows a separation of the OPA–ethanethiol derivatives of seven biogenic amines including pentylamine as the internal standard using a mobile phase of 85% (v/v) methanol and 15% 84 mmol/l triethylamine–acetic acid pH 7.5. A high concentration of the organic base is essential in order to keep the peak of histamine as narrow as possible. For instance, at a 30 mmol/l concentration, the histamine peak emerges with a broad base, overlapping partially the peak of tyramine (not shown).

By performing derivatisation in a system consisting of both an aqueous buffer and an organic solvent, the derivatives formed remain distributed within the organic supernatant only. Under such conditions, storage properties of the derivatives were examined by determining their values at 1 and at 24 h after derivatisation. The former and the latter values were taken as the control (100% values) and percentage recoveries 24 h after derivatisation, respectively. Recoveries in butanol, EMK–isopropanol or EMK supernatants, respectively, were for histamine 88, 103 and 102%, and for the other amines between 93 and 106%, irrespective of the solvents. According to these results, the derivatives exhibit marked stability under the conditions used, and they may be measured by HPLC without timing of the derivatisation, as is commonly required when derivatisations with OPA are performed in aqueous solutions only.

In efforts to identify biogenic amine-producing cultures of bacteria, we have been concentrated on those producing either histamine or tyramine as the most important representative of vasoactive or psychoactive amines, respectively. Fig. 2 shows chromatograms, which were derived from

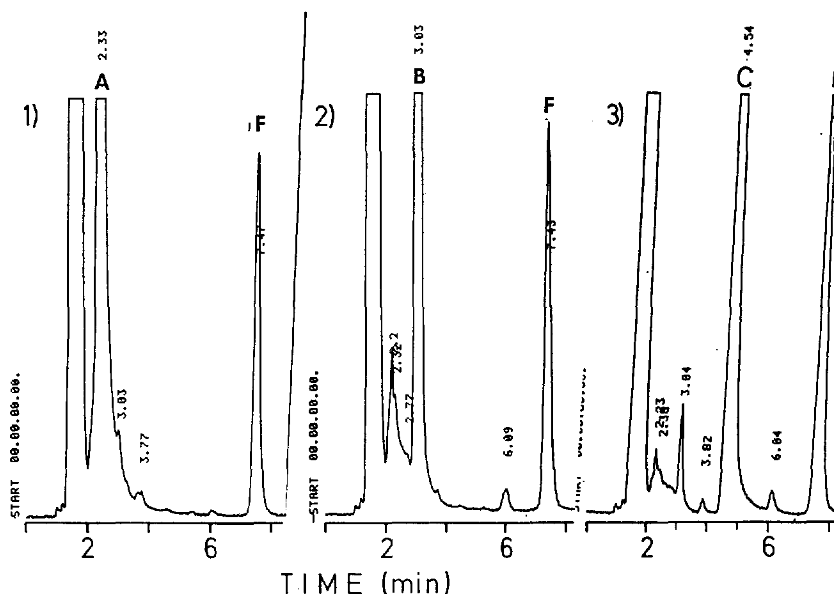

Fig. 2. Separation of amines extracted from a 10-μl volume of a bacterial culture producing either histamine (chromatogram 1), tyramine (chromatogram 2) or tryptamine (chromatogram 3). Peaks: A = histamine, B = tyramine, C = tryptamine, and F = pentylamine. Separation conditions as in Fig. 1.

bacterial cultures accumulating either histamine (chromatogram 1), tyramine (chromatogram 2) or tryptamine (chromatogram 3) at a concentration of 11, 9.5 or 13 mmol/l, respectively. The tryptamine-producing culture was accidentally detected, and is shown here for illustration purposes.

In order to demonstrate detection of cultures producing amines other then those presented, a grown tyramine-producing culture was analysed for biogenic amines prior to and after spiking with seven biogenic amines at a concentration of 1 mmol/l each. Results are shown in Fig. 3. Although added at a 10-fold smaller concentration then usually seen in cultures, all the biogenic amines are clearly visible in the chromatogram. Note that in this case extraction was performed with EMK.

Common procedures for extraction of biogenic amines from a variety of biological materials include mostly a deproteinisation step with mineral acids only [8,9,12,13] or are followed by either ion-exchange clean-up at pH 4.5 or 6.0 including an acidic elution step of the amines [10,11,18,19]) or butanol–chloroform extraction for a period of 30 min at pH 12.0 [15]. All these techniques are either too laborious for screening applications or provide insufficient clean-up of samples.

The present procedure involves aseptic sampling of a broth culture by transferring a 10-μl volume onto the Extrelut packing, followed by extraction with either EMK–isopropanol or EMK. The extraction step is rapid and requires a few minutes only. Such extracts are suitable for both OPA derivatisation and isocratic HPLC.

Extraction efficiency was examined on Extrelut packings by determining extraction rates of biogenic amines in the eluate, expressed as a percentage, using a variety of solvents. The following values were recorded in butanol, EMK–isopropanol or EMK supernatants, respectively: for histamine 90, 61 and 38%, putrescine 44, 28 and 23%, cadaverine 79, 36 and 33% and for tyramine, tryptamine, isobutylamine, phenethylamine and pentylamine in the range of

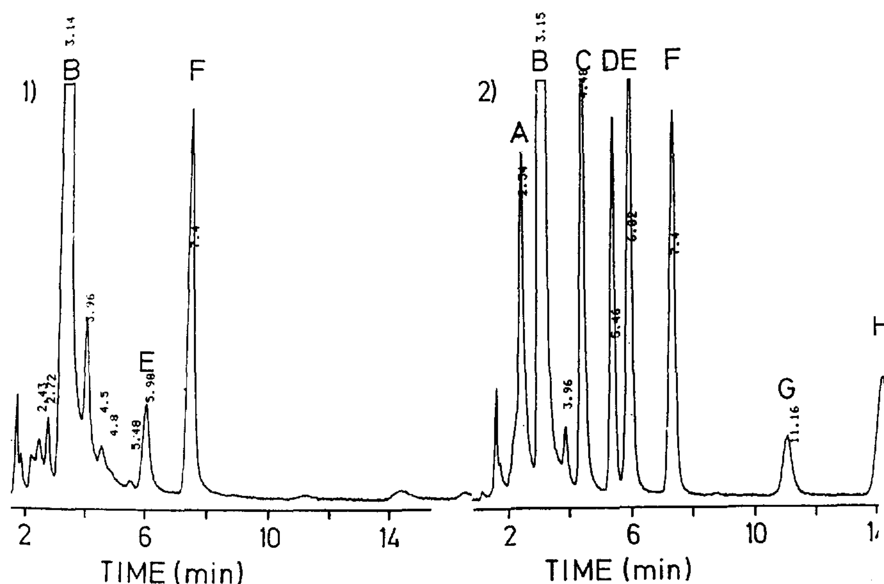

Fig. 3. Separation of biogenic amines from a broth cultured prior to [chromatogram 1 with peaks of tyramine (B), pentylamine (F) and phenethylamine (E)] and after spiking with biogenic amines [chromatogram 2 with peaks of histamine (A), tyramine (B), tryptamine (C), isobutylamine (D), phenethylamine (E), pentylamine (F), putrescine (G) and cadaverine (H)]. Separation conditions as in Fig. 1.

79–95%, 65–84%, and 52–80%, respectively. Also, butanol–chloroform (90:10, v/v) was used. However, extraction rates were less then 2.5%, possibly because of the short extraction times used.

The highest extraction rates were obtained with butanol. However, this solvent gave excessive front peaks in chromatograms, which may overlap the nearby histamine (not shown), and is therefore unsuitable for the present purposes. However, levels of biogenic amines in cheese sold under the commercial name of the Swiss green cheese (der Glarner Kreuterkäse) were measured successfully using butanol extraction. In this case, 5% homogenates were prepared in sodium ascorbate–phosphate pH 12.5, and 20-μl aliquots were extracted on Extrelut packings.

When large numbers of cultures were to be assayed for either histamine or tyramine production, steps of extraction and derivatisation were performed in series of ten samples. Also, use of internal standards was often omitted, in order to keep separation times as short as possible. According to our experience, one bacterial strain produces usually one amine only. Among 500 amine-producing strains isolated, only one strain was found to produce histamine, isobutylamine and isopentylamine. However, this strain was classified as non-dairy-related.

4. Conclusion

The present procedure has been developed primarily for screening purposes in order to identify those cultures producing biogenic amines in cheese. Under the conditions used, the derivatives of biogenic amines obtained with OPA–ethanethiol are markedly stable, and meet the requirements for HPLC separation. Putrescine and cadaverine values have been taken as approximate amounts, because of their low extraction rates. The majority of amines may be estimated reliable with recoveries of added amounts ranging from 74 to 96%.

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