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Note

Quantitative determination of free volatile fatty acids from dairy products on a Nukol capillary column

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Free volatile fatty acids (FVFA) in dairy products are produced in only small amounts by lipolytic processes, and are derived substantially from amino acid degradations and lactose fermentation. For this reason, FVFA concentrations show a trend that varies progressively during ripening. As a consequence, the qualitative and quantitative evaluation of these components could both contribute to the characterization of various kinds of cheeses and enable one to relate chemical composition to ripening level for each type of product [1–11].

Determination of FVFA is usually carried out by gas chromatography (GC). In early studies, methods employing packed columns were proposed for the determination of the analytes either in the free form, by utilizing stationary phases such as silicone oil–stearic acid mixtures [12–16], Tween 80 [15,17,18], diethylene glycol adipate (DEGA)–H_3PO_4 [19,20], free fatty acid phase (FFAP) [21–23], Porapak Q H_3PO_4 [24–27], diethylene glycol succinate (DEGS)–H_3PO_4 [28], NPGA H_3PO_4 [29,30], Chromosorb 101 [31–33] or SP-1200–H_3PO_4 [34–36], or after conversion into the corresponding methyl [37–40], butyl [41,42], decyl [41], benzyl [43,44], phenacyl [41] or *p*-bromobenzyl [45] esters. More recently, methods employing capillary columns have been proposed [46–49].

In this paper, a method for the determination of C_2–C_8 free carboxylic acids in dairy products is described, involving use of a Nukol capillary column and crotonic acid as the internal standard.

EXPERIMENTAL

Chemicals

Acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, crotonic, caproic and caprylic acids were purchased from Fluka and were all standards for gas chromatography (>99% purity, except isovaleric acid, which was >98%). Isocaproic acid was obtained from Sigma and was *ca.* 99% pure.

The corresponding sodium salts were prepared by neutralization of acetone solutions of the free acids with 0.1 *M* methanolic sodium hydroxide, dried in a rotary

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vacuum evaporator at 40°C, powdered in a mortar, transferred to screw-stoppered vials and kept in a desiccator over phosphorus pentoxide.

Formic acid was obtained both from BDH (90% pure) and from Carlo Erba (99% pure). Lactic, pyruvic and 2-oxobutyric acids were obtained from Fluka.

Preparation of standard solutions

A stock standard FVFA solution was prepared by accurately weighing *ca.* 500 mg each of acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, caproic and caprylic acids in a 25-ml glass-stoppered volumetric flask and diluting to the mark with dichloromethane. By successive 1:10 dilutions with dichloromethane, the working standard FVFA solutions were prepared. Stock and working standard crotonic acid (internal standard) solutions used were prepared in a similar manner. The final concentrations of each component for all the standard solutions prepared are given in Table I.

Gas chromatography

A Carlo Erba HRGC 5300 Mega gas chromatograph equipped with a flame ionization detector was used. A Nukol fused-silica capillary column (15 m × 0.53 mm I.D.) with a film thickness of 0.5 μm (Supelco) was employed. The column temperature was programmed from 100 to 180°C at 10°C/min. Cold on-column injection was used. The detector temperature was 200°C, carrier gas (hydrogen) flow-rate 15 ml/min (set at 100°C), detector attenuation 10 and sample size 1 μl. Peak areas were determined with a Carlo Erba Mega integrator.

Calibration

A 5-ml volume of working standard FVFA solution and 5 ml of working standard crotonic acid solution were transferred into a 20-ml glass-capped tube over anhydrous sodium sulphate and 1 μl was injected immediately into the column.

Various combinations of working standard FVFA and crotonic acid solutions allowed amounts of each acid from 10^{-6} down to 10^{-9} g to be injected and 100:1, 10:1,

TABLE I

FINAL CONCENTRATIONS OF EACH COMPONENT IN THE STANDARD SOLUTIONS PREPARED

Standard solution	Concentration of each component (mg/l)
Stock FVFA solution	20000
Working FVFA solution 1	2000
Working FVFA solution 2	200
Working FVFA solution 3	20
Working FVFA solution 4	2
Stock crotonic acid solution	20000
Working crotonic acid solution 1	2000
Working crotonic acid solution 2	200
Working crotonic acid solution 3	20
Working crotonic acid solution 4	2

1:1, 1:10 and 1:100 ratios between the amount of each FVFA and the amount of crotonic acid to be obtained. Five analyses were performed for each determination.

Conversion of the sodium salts into the corresponding free acids

Standard FVFA sodium salts suspension (SSS). Amounts of acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, crotonic, caproic and caprylic acid sodium salts (corresponding to about 10 mg each of free acid) were accurately weighed in a 25-ml glass-stoppered volumetric flask, diluted to the mark with dichloromethane and 20 μl of phosphoric acid (Merck, 85%) were added. The flask was kept on a magnetic stirrer overnight, then the solids were allowed to settle.

1-Dodecanol solution. A 6.3-mg amount of 1-dodecanol (Fluka, >99% pure) was accurately weighed in a 25-ml glass-stoppered volumetric flask and diluted to the mark with dichloromethane.

Procedure. A 500-μl volume of clear solution from the SSS and 500 μl of the 1-dodecanol solution were transferred into a 5-ml glass-capped tube over anhydrous sodium sulphate, and 1 μl of the solution obtained was injected immediately into the column.

Both the SSS and the 1-dodecanol solutions were diluted again to the mark with dichloromethane, and to the SSS 20 μl of phosphoric acid were added, the flask was kept on a magnetic stirrer overnight and, after the solids had settled, the procedure was repeated from the beginning. With successive 20-μl additions in this manner, amounts of phosphoric acid in the range up to 120 μl were examined. Five analyses were performed for each determination.

Determination of FVFA in cheese

A 100-g amount of a 15 day-old Montasio cheese was steam distilled as described elsewhere [10,11]. The distillate was neutralized with 0.1 *M* sodium hydroxide solution, using phenolphthalein as the indicator, and mixed with 5 ml of a 250 mg/l solution of sodium crotonate. The sodium salts were dried on a rotary vacuum evaporator at 40°C. The dry salts (*ca.* 130 mg) were converted into the corresponding free acids by adding 10 ml of a solution of phosphoric acid in dichloromethane (20 mg/l); the solution obtained was transferred into a 20-ml glass-capped tube over anhydrous sodium sulphate, and 1 μl was injected immediately into the column.

RESULTS AND DISCUSSION

One of the most widely used methods for the determination of FVFA in dairy products involves preliminary recovery from the matrix by steam distillation, followed by GC separation [1,5,7,9–11]. However, relatively high distillate volumes have to be collected in order to achieve a quantitative yield of all the compounds of interest [10,11]. As a consequence, a solution in which FVFA are present at lower concentrations than in the starting product is usually obtained by steam distillation. For this reason, the distillate has to be neutralized and water eliminated, thus obtaining the salts of the corresponding acids. By adding a strong acid, the salts are reconverted into the free acids before GC separation.

Recently, a wide-bore Nukol column has become commercially available and seems very attractive for the determination of FVFA in dairy products. A wide-bore

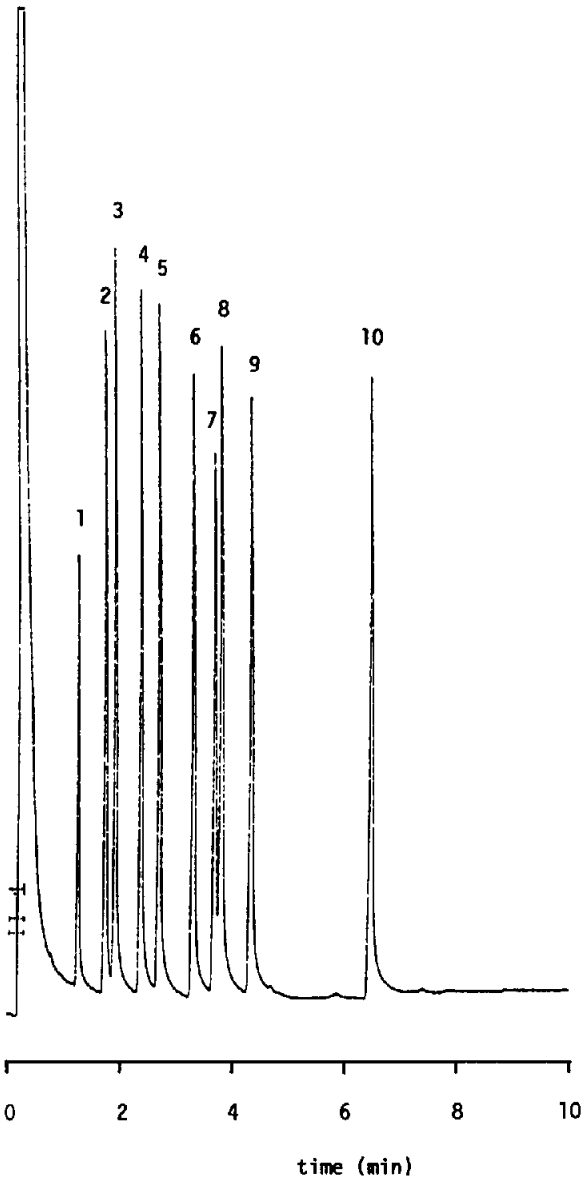

Fig. 1. GC separation on a Nukol capillary column of a synthetic mixture of volatile fatty acids. Peaks: 1 = acetic acid; 2 = propionic acid; 3 = isobutyric acid; 4 = *n*-butyric acid; 5 = isovaleric acid; 6 = *n*-valeric acid; 7 = crotonic acid; 8 = isocaproic acid; 9 = caproic acid; 10 = caprylic acid.

column has the advantages of both a packed and a capillary column (high capacity, high sensitivity, short analysis time, high resolving power). It is therefore possible to inject a mixture in which the components are present in amounts that are notably different from one another (a situation typical of FVFA recovered from dairy products), and to obtain an excellent separation in a short time.

As an example, Fig. 1 shows the separation of a synthetic mixture of all the C_2–C_8 FVFA usually present in dairy products, with the addition of crotonic acid as a suitable internal standard. The responses of the individual FVFA are linear over a wide range of injected amounts (see Fig. 2).

FVFA are present in cheeses in amounts usually ranging from about 100 to about 0.01 mg per 100 g. It is therefore possible to use an amount of internal standard of 1 mg per 100 g of product for the determination of all the compounds of interest, by utilizing a calibration graph of the type shown in Fig. 3.

Formic acid has been suggested for the conversion of the salts into the corresponding free acids [9,50–54]. However, it was not possible to use formic acid here because, unexpectedly, the injection of a solution of formic acid in dichloromethane into the Nukol column produced a peak that interferes with, and may obscure, the propionic acid peak. The phenomenon was observed both on utilizing formic acid from different commercial sources and on passing the solution of formic acid even over a large excess of anhydrous sodium sulphate. In contrast, the injection of a solution of phosphoric acid in dichloromethane gave a chromatogram completely free from

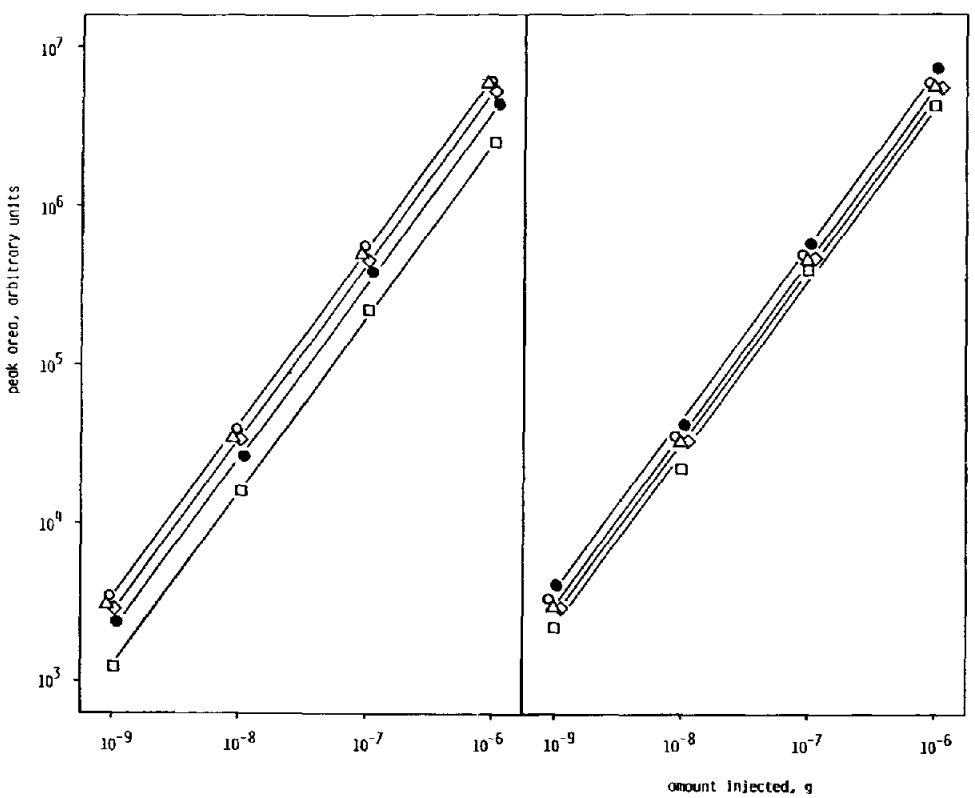

Fig. 2. Calibration graph of amount of each volatile fatty acid injected *versus* detector response (average of five determinations). Left: □ = acetic acid; ● = propionic acid; ◇ = isobutyric acid; ○ = isocaproic acid; △ = caproic acid. Right: ◇ = *n*-butyric acid; ○ = isovaleric acid; △ = *n*-valeric acid; □ = crotonic acid; ● = caprylic acid.

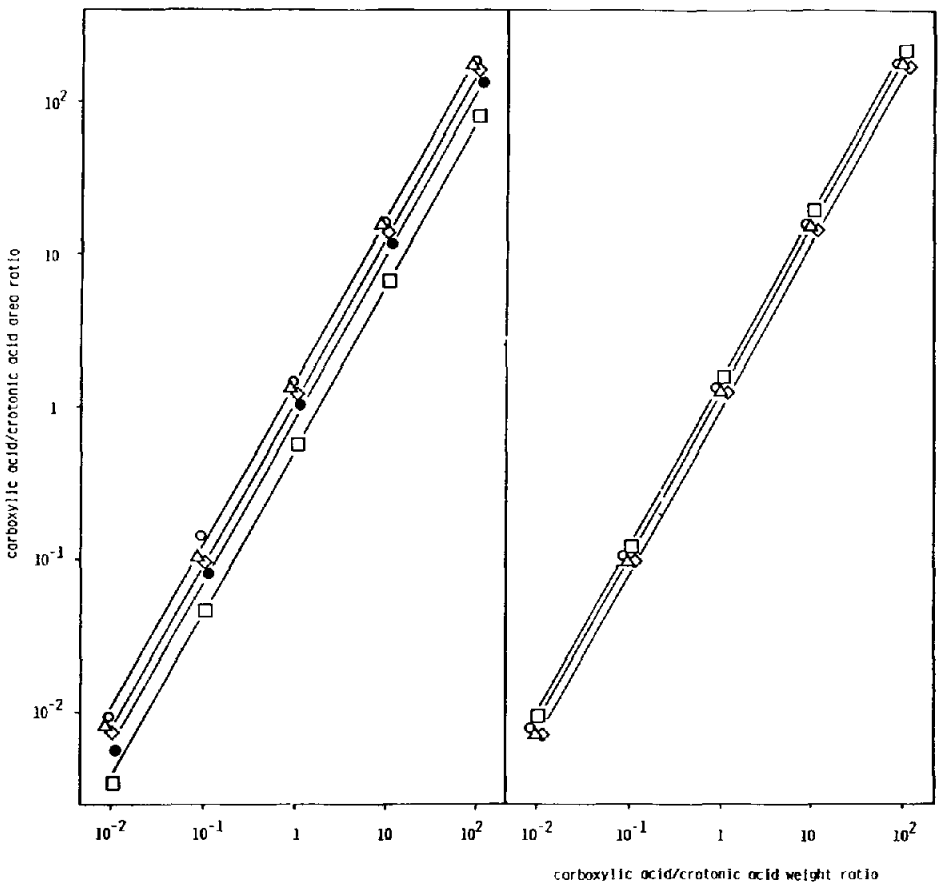

Fig. 3. Calibration graph of peak-area ratio *versus* weight ratio for each volatile fatty acid with respect to crotonic acid as the internal standard (average of five determinations). Left: □ = acetic acid; ● = propionic acid; ◇ = isobutyric acid; ○ = isocaproic acid; △ = caproic acid. Right: ◇ = *n*-butyric acid; ○ = isovaleric acid; △ = *n*-valeric acid; □ = caprylic acid.

interfering peaks. The addition of phosphoric acid to the salts in order to obtain the FVFA sample to be injected was been reported previously [20,22,26,27,55–59]. The possibility of using phosphoric acid for the conversion of the salts into the corresponding free acids was then investigated. In particular, the best ratio between the amount of salts and the amount of phosphoric acid was studied, in order to obtain a quantitative yield of all the acids of interest. For this purpose, increasing amounts of phosphoric acid were added to a synthetic mixture of sodium salts; the conversion of the salts into the corresponding free acids was evaluated by utilizing 1-dodecanol as an internal standard. The free carboxylic acid-to-1-dodecanol peak-area ratios were used to evaluate the completeness of the conversion.

The results obtained, presented in Fig. 4, show that a 1:2 (v/w) ratio of phosphoric acid to the salts is sufficient to obtain a complete conversion into the free

acids; an amount of phosphoric acid even twice the minimum necessary gives rise to no undesirable effects.

It is interesting that the addition of phosphoric acid leads to the conversion first of the branched-chain, then to the straight-chain and finally to the unsaturated acids.

The method was applied to the determination of FVFA in a Montasio cheese (Fig. 5). An excellent separation of the C_2–C_8 FVFA was obtained even with respect to lactic acid, which is recovered by steam distillation from dairy products together with the volatile acids of interest. Oxo acids do not interfere, as they are not eluted from the Nukol column, as was verified by injecting pure standards of pyruvic and 2-oxobutyric acids.

No ghosting effect was observed on the Nukol column on injecting a 5 g/l solution of phosphoric acid in dichloromethane even just after a mixture of 1 g/l of each FVFA.

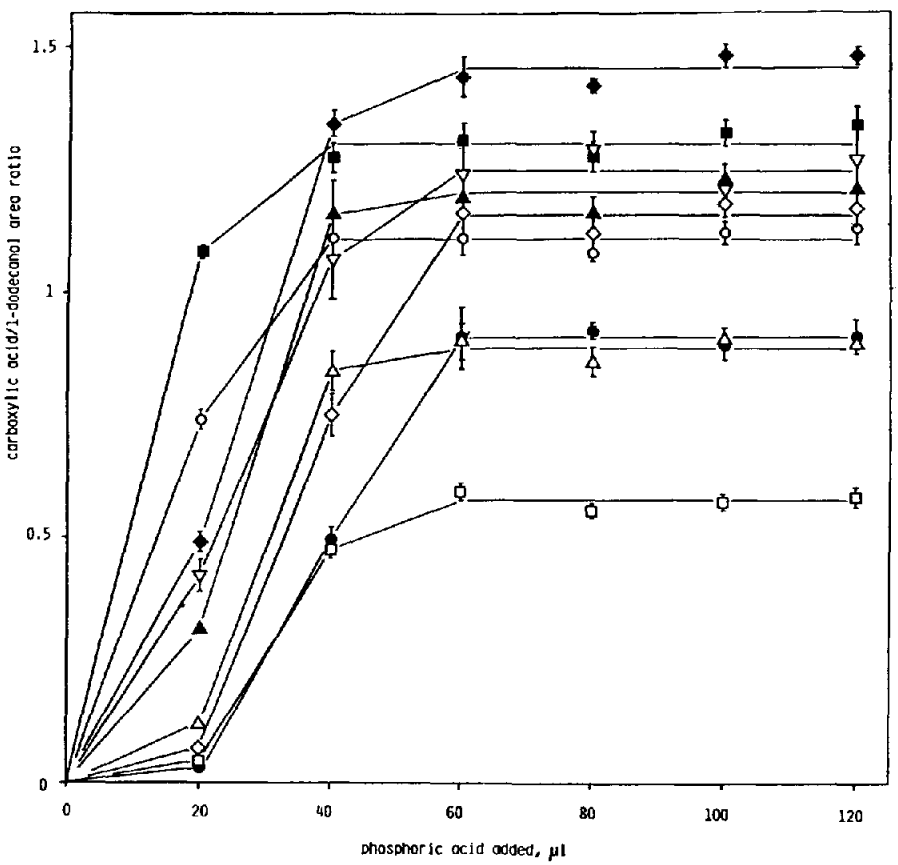

Fig. 4. Evaluation of the conversion of sodium salts into the corresponding free acids by addition of phosphoric acid, using 1-dodecanol as the internal standard (average of five determinations ± S.D.). □ = Acetic acid; △ = propionic acid; ○ = isobutyric acid; ◇ = *n*-butyric acid; ■ = isovaleric acid; ▲ = *n*-valeric acid; ● = crotonic acid; ◆ = caproic acid; ▽ = caprylic acid.

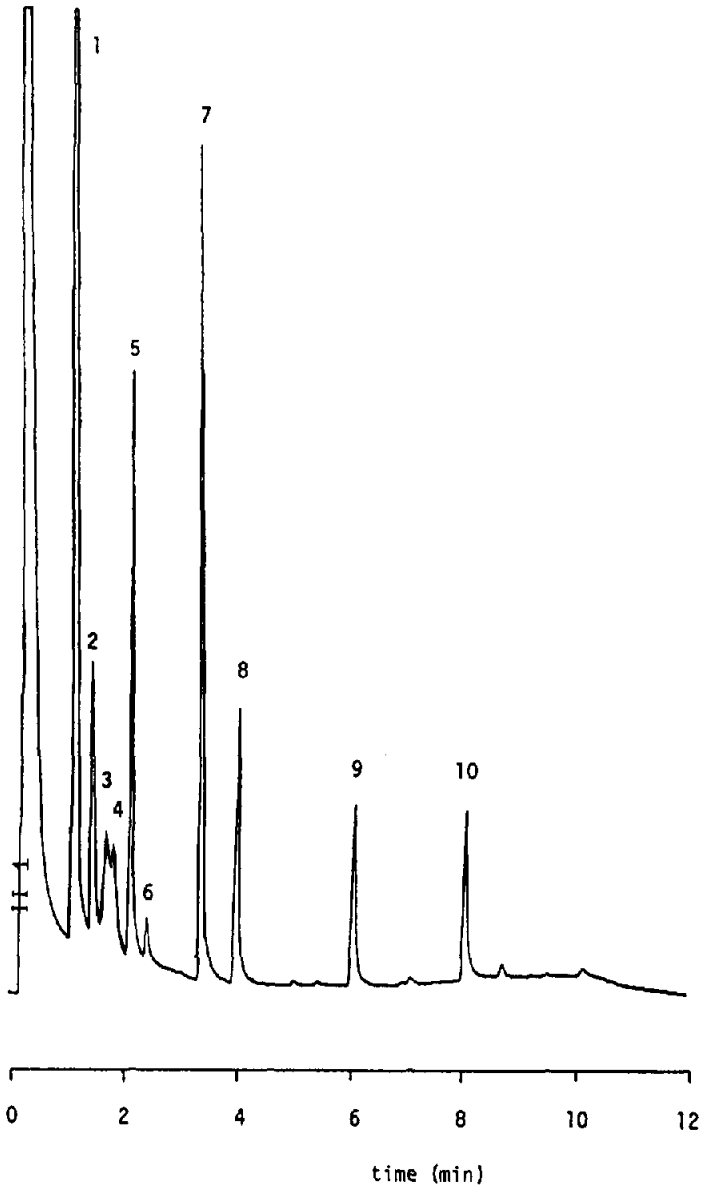

Fig. 5. GC separation on Nukol capillary column of free volatile fatty acids recovered from a Montasio cheese, with the addition of crotonic acid as the internal standard. Peaks: 1 = acetic acid; 2 = propionic acid; 3 = isobutyric acid; 4 = unknown; 5 = *n*-butyric acid; 6 = isovaleric acid; 7 = crotonic acid; 8 = caproic acid; 9 = caprylic acid; 10 = lactic acid.

No apparent sign of deterioration of the column performance, such as tailing or the appearance of spurious peaks, was observed after 6 months of continuous use.

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