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SEPARATION OF SATURATED, MONO-UNSATURATED AND DI-UNSATURATED ALDEHYDES AS 2,4-DINITROPHENYLHYDRAZONES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AT INCREASED TEMPERATURE*

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SUMMARY

Saturated and mono- and di-unsaturated aldehydes (C_5–C_{10}) were converted into their 2,4-dinitrophenylhydrazones. The separation of nanogram amounts of these hydrazones was performed by high-performance liquid chromatography with C_{18} reversed-phase columns in an isocratic elution. Separation of all aldehydes tested could only be achieved at an elevated temperature (50°C). A linear relationship between log k' and the carbon number for a series of homologous compounds could be observed also at an elevated temperature. An optimal separation was achieved utilizing the methylene group selectivity which was found to be temperature dependent.

Application of this method shows the advantage of an optimized separation for the determination of carbonyl compounds from the oxidation of fatty acids in meat.

INTRODUCTION

Aldehydes and other carbonyl compounds are ubiquitous in the environment. In addition to the presence of carbonyl compounds in air and water, their presence in foods is of considerable interest. Owing to their extremely low olfactory and gustatory threshold concentrations they contribute essentially to the aroma of foods even if present in trace amounts only. This is particularly true in respect of fat-containing foods in which the oxidation of fatty acids will result in the formation of carbonyls and other compounds. For the demonstration of such trace concentrations, a sensitive method of measurement is required.

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Two different methods are commonly used for the analytical detection of aldehydes in low concentrations as present in foods. One consists of isolation of the carbonyl compounds by extraction or distillation and subsequent gas chromatographic determination without derivative formation[1–3]. The other consists of reaction with 2,4-dinitrophenylhydrazine (2,4-DNP) and chromatographic determination of the hydrazones.

Gas chromatographic determination also records numerous volatile accompanying substances since the flame-ionization detector which is commonly used does not indicate carbonyl compounds in a selective or specific way. Beyond this, its sensitivity is mostly insufficient for trace analysis. The spectrum of aldehydes may be reliably determined from a biological matrix only by a combination of gas chromatography and mass spectrometry. By reacting with 2,4-DNP, the carbonyl compounds are converted into derivatives which owing to their characteristic absorption at 340–410 nm can be clearly distinguished from the accompanying substances. Because of their high molar absorptivities, 2,4-dinitrophenylhydrazones (2,4-DNPHs) are well suited for the detection of traces[4].

A number of chromatographic methods for the separation of 2,4-DNPHs of carbonyl compounds have been described. Gas chromatographic separation of 2,4-DNPHs has been performed by a number of authors[5–8]. When compared to separation methods using thin-layer and liquid chromatography, this method will, however, show obvious disadvantages. Separation of aldehydes by multiple thin-layer chromatography (TLC) on various coating materials has been described[9]. Separation of aldehyde classes has been achieved on Kieselguhr hydrophobized with Carbowax 400[10–12]. Separation of 2,4-DNPHs by liquid chromatography has been conducted on Corasil[13,14]. The use of high-performance liquid chromatography (HPLC) with reversed-phase columns results in a better separation. Selim[15] and Fung and Grosjean[4] have reported a determination of C_1–C_6 aldehyde hydrazones in C_{18} reversed-phase columns with acetonitrile–water mixtures using isocratic elution. Demko[16] and Nakamura *et al.*[17] used gradient elution for the separation of straight-chain saturated C_1–C_{10} aldehyde hydrazones with acetonitrile–water mixtures. Vigh *et al.*[18] have described the separation of saturated (C_1–C_{12}) as well as of mono-unsaturated (C_3–C_8) aldehyde hydrazones using methanol–water mixtures with isocratic elution. The resolution of the separation achieved between saturated and mono-unsaturated aldehyde hydrazones did not appear to be satisfactory.

In this paper, an HPLC method is described which permits a separation of hydrazones from saturated and mono- and di-unsaturated aldehydes. In particular it permits a quantitative determination of all the aldehydes characteristic of the formation of off-flavour in foods at the relevant trace level.

EXPERIMENTAL

Materials

Chemicals were of analytical-reagent grade unless indicated otherwise. Pure water was obtained using Millipore equipment.

2,4-Dinitrophenylhydrazine (2,4-DNP), 95% ethanol and 98% sulphuric acid were purchased from E. Merck. Hexanal was obtained from Fluka, pentanal, 2-*trans*-hexenal, 2,4-heptadienal, 2-*trans*-heptenal, heptenal, 2-octenal, octanal, 2,4-non-

adienal, 2-nonenal, nonanal, 2,4-decadienal and 2-*trans*-decenal from Atlanta and decanal from Chrompack. Acetonitrile, tetrahydrofuran and methanol were obtained from E. Merck. ChromAR water for HPLC was purchased from Prochem. G.F.R.). All solvents were filtered using a 0.7-μm fibre glass filter.

Preparation of 2,4-DNPH standards

2,4-DNP (4 g) was dissolved in 10% sulphuric acid and the aldehyde (1.5 ml) was then added. The precipitated 2,4-DNPH was separated after 1 h and recrystallized twice from ethanol. Purity was checked by HPLC.

HPLC

The HPLC equipment consisted of a Spectra-Physics 8700 HPLC pump with helium eluent degassing, a Kipp Model 9209 automatic sample injector with a Rheodyne No. 70-10 sample valve, a Knauer type 89.00 column oven and a Perkin-Elmer LC 55 variable-wavelength detector. An HP 3390 A integrator was used for the calculation of peak areas.

The 2,4-DNPHs were separated on a 250 × 4.6 mm I.D. Supelcosil LC 18 column, 5 μm (C_{18} reversed-phase) with isocratic elution at 1.0 ml/min. Between the injector and main column a 30 mm guard column with LiChrosorb RP-18, 5 μm, was used. The eluent was acetonitrile–water–tetrahydrofuran (75:24:1, v/v/v). The eluent was pre-heated in a 100-mm LiChroprep RP-18 column installed between the pump and the injector. The temperature of all columns was 50°C. The injected volume was 20 μl and detection took place at 360 nm.

RESULTS AND DISCUSSION

The chromatographic system was designed for a complete separation of all the aldehydes expected as products of the autoxidation of lipids. The aldehydes with carbon numbers 1–4 were not included as these are less indicative of autoxidation of fatty acids and are less important as components of the off-flavour. On the other hand, the solvents used are largely contaminated with these low-chain aldehydes, which may interfere if not removed completely during the purification procedure.

Fig. 1 shows the chromatogram of the test mixture containing the 2,4-DNPHs of all the relevant aldehydes. All the aldehydes could be separated, allowing identification by means of retention times. The separation of pentanal (C_5), hexanal (C_6), 2-heptenal ($C_{7:1}$) and heptanal (C_7) was complete, with a resolution of $R \geq 1.4$. The group 2-octenal ($C_{8:1}$) 2,4-nonadienal ($C_{9:2}$) and octanal (C_8) and the pair 2-nonenal ($C_{9:1}$) and 2,4-decadienal ($C_{10:2}$) were separated with a resolution $R \approx 0.8$. This means for practical purposes that the aldehydes can be determined quantitatively with an electronic integrator. This has been confirmed by many routine analyses measuring autoxidation in meat samples.

The chromatographic system as presented in Fig. 1 is the result of an optimization with regard to resolution and time needed for analysis by changing the eluent and the temperature. All fractions are eluted with capacity factors k' between 1.8 and 8.6. Addition of 1% of tetrahydrofuran as a modifier resulted in a considerable sharpening of the eluting peaks. An increase in temperature from room temperature

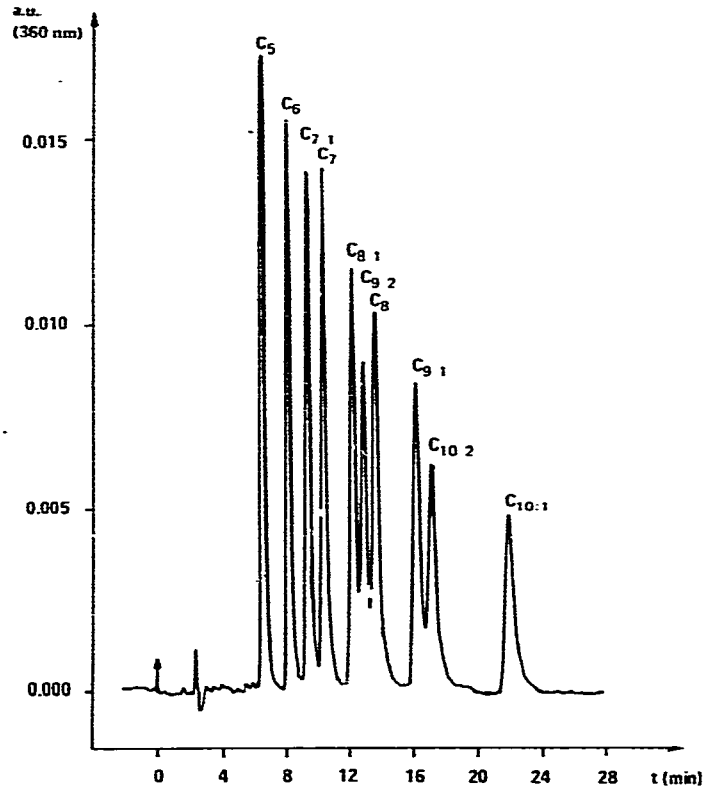

Fig. 1. HPLC trace for 2,4-DNPHs of ten aliphatic aldehydes on a C_{18} reversed-phase column (pentanal 100 ng, all others 80 ng).

(25°C) to 50°C resulted in a decisive improvement of the separation between C_8 and $C_{9:2}$ and a shortening of the time needed for a run, as shown in Fig. 2.

As can be seen from Fig. 1, mono-unsaturated aldehydes are eluted before saturated aldehydes of the same chain length. This effect increases when there is a second double bond in the molecule and can be explained by the unsaturated compounds being more polar than the saturated ones. It is obvious that this can lead to interferences in complex mixtures.

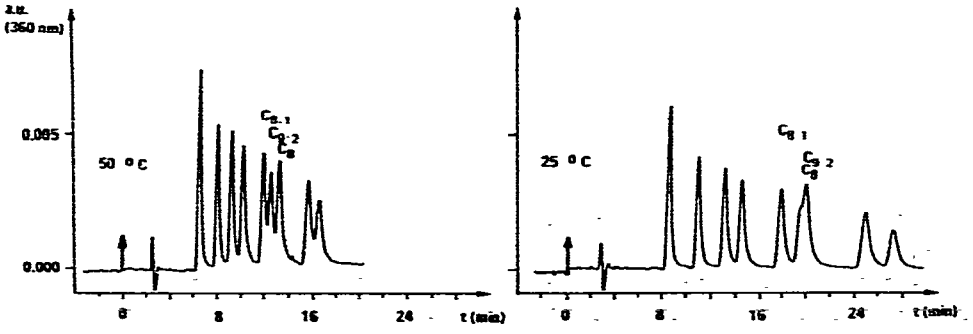

Fig. 2. HPLC trace for 2,4-DNPHs of nine aliphatic aldehydes at two column temperatures.

In Fig. 3, the relationship between chain length, degree of unsaturation and retention volume is plotted in a semi-logarithmic diagram. The members of the homologous series of saturated, mono-unsaturated and di-unsaturated aldehydes demonstrate a logarithmic increase in the capacity factor k' as a function of the carbon number. The correlation between the carbon number and log k' is found to be linear for all series. The lines in Fig. 3a and b obtained from data at both temperatures are parallel with each other within the accuracy of the measurements. These log k' graphs are suitable for predicting the retention value of other not readily available members of the homologous series. These can be further characterized by their absorption spectra which for various homologous series exhibit maxima dependent on the degree of unsaturation.

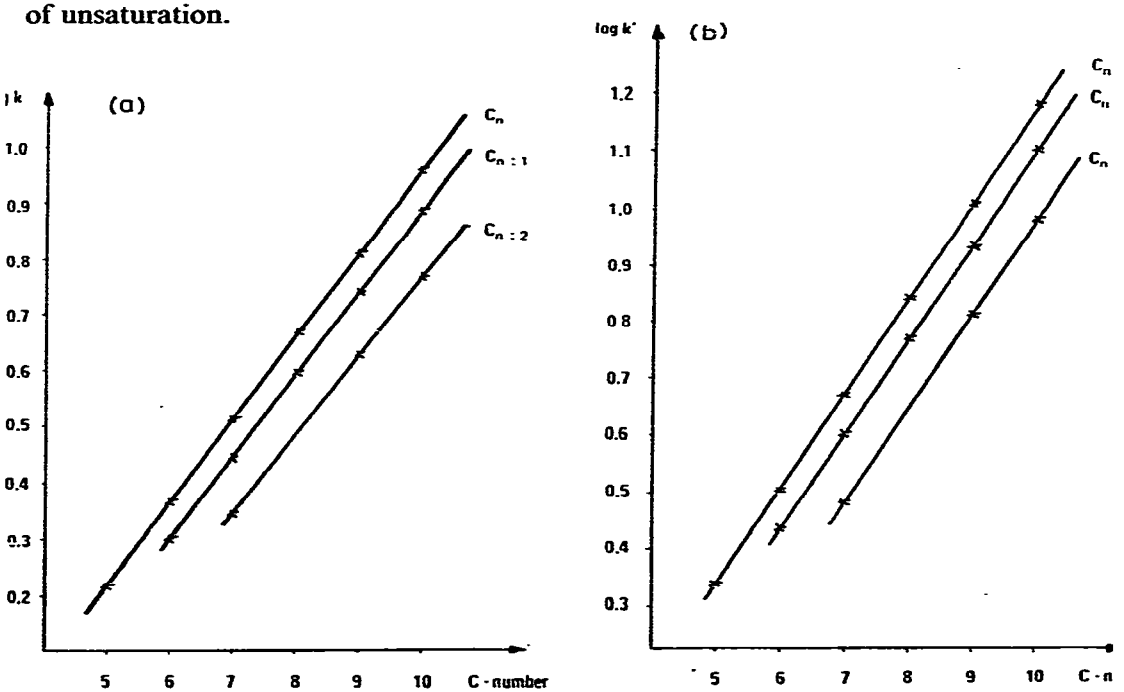

Fig. 3. Relationship between carbon number of aliphatic, straight-chain, saturated and mono- and di-unsaturated aldehydes and log k' at column temperatures (a) 50 and (b) 25°C.

At 25°C, the di-unsaturated aldehydes are eluted very close to the saturated aldehydes with one carbon less. At 50°C, all aldehydes were eluted separately. The different elution patterns at the two temperatures can be regognized from the two graphs in Fig. 3a and b. A more extended study of the influence of the column temperature on log k' and the separation of critical triplets formed by aldehydes of the saturated and mono- and di-unsaturated series is summarized in Fig. 4. From the diagram it is obvious that at higher temperatures di-unsaturated aldehyde hydrazones are accelerated more than the two other groups. This was found to be true for three critical triplets studied, of which two are shown in Fig. 4. Another effect of raising the temperature is an increase in column efficiency resulting in sharper peaks and shorter analysis times. These observations make it clear that separation of the critical triplets of aldehydes may be achieved by optimization of the temperature. For the case of

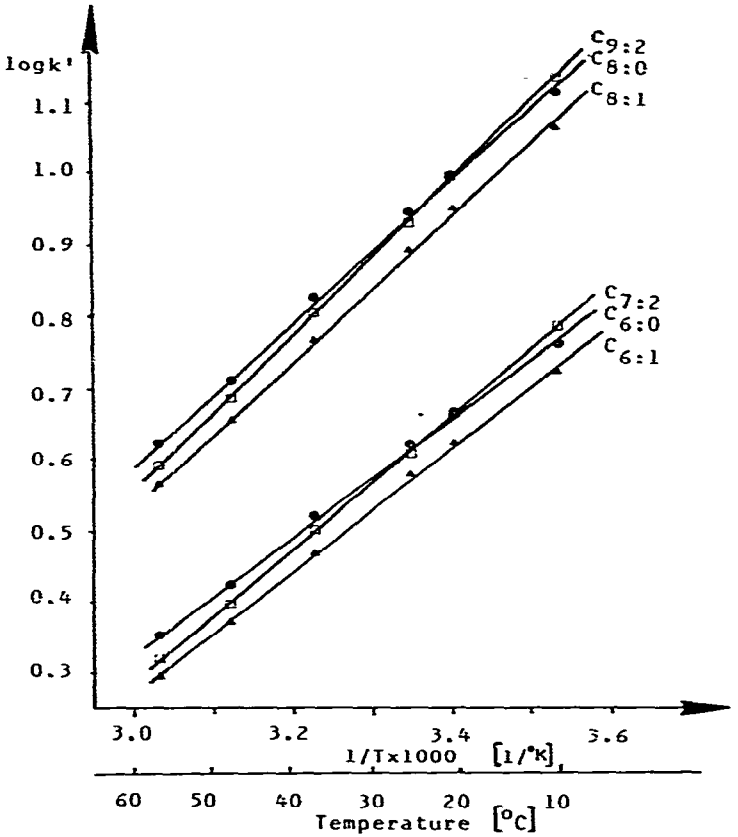

Fig. 4. Influence of the column temperature on log k' of critical triplets of aldehydes (log k' values in this figure are not the same as those in Fig. 3a and b because another column with the same packing material was used).

autoxidation of lipids, the critical triplet of practical importance is C_8, $C_{8:1}$ and $C_{9:2}$, which is separated best at 50°C (Figs. 2 and 4).

As mentioned previously, the separation system was optimized to determine the aldehydes formed during autoxidation of food samples. In Fig. 5, two chromatograms are reproduced to demonstrate the practical utility of the method. The chromatogram in Fig. 5a clearly shows the absence of aldehydes in a fresh pork liver sample with the exception of a small amount of hexanal. The chromatogram in Fig. 5b, in contrast, was obtained from a sample of identical origin after storage for 6 months at −8°C. A large hexanal peak dominates the chromatogram accompanied by the whole series of aldehydes at low concentrations as would be expected for autoxidation of animal lipids. This chromatogram indicates clearly strong rancidity. In order to give some idea about the sensitivity of detection in food samples it should be noted that the hexanal peak in Fig. 5a corresponds to 40 ppb and the peaks for octenal and heptenal in Fig. 5b represent concentrations of 90 and 20 ppb, respectively. This shows that under routine conditions aldehydes can be detected in meat samples in the lower ppb range.

The method described has been developed for the investigation of the progress

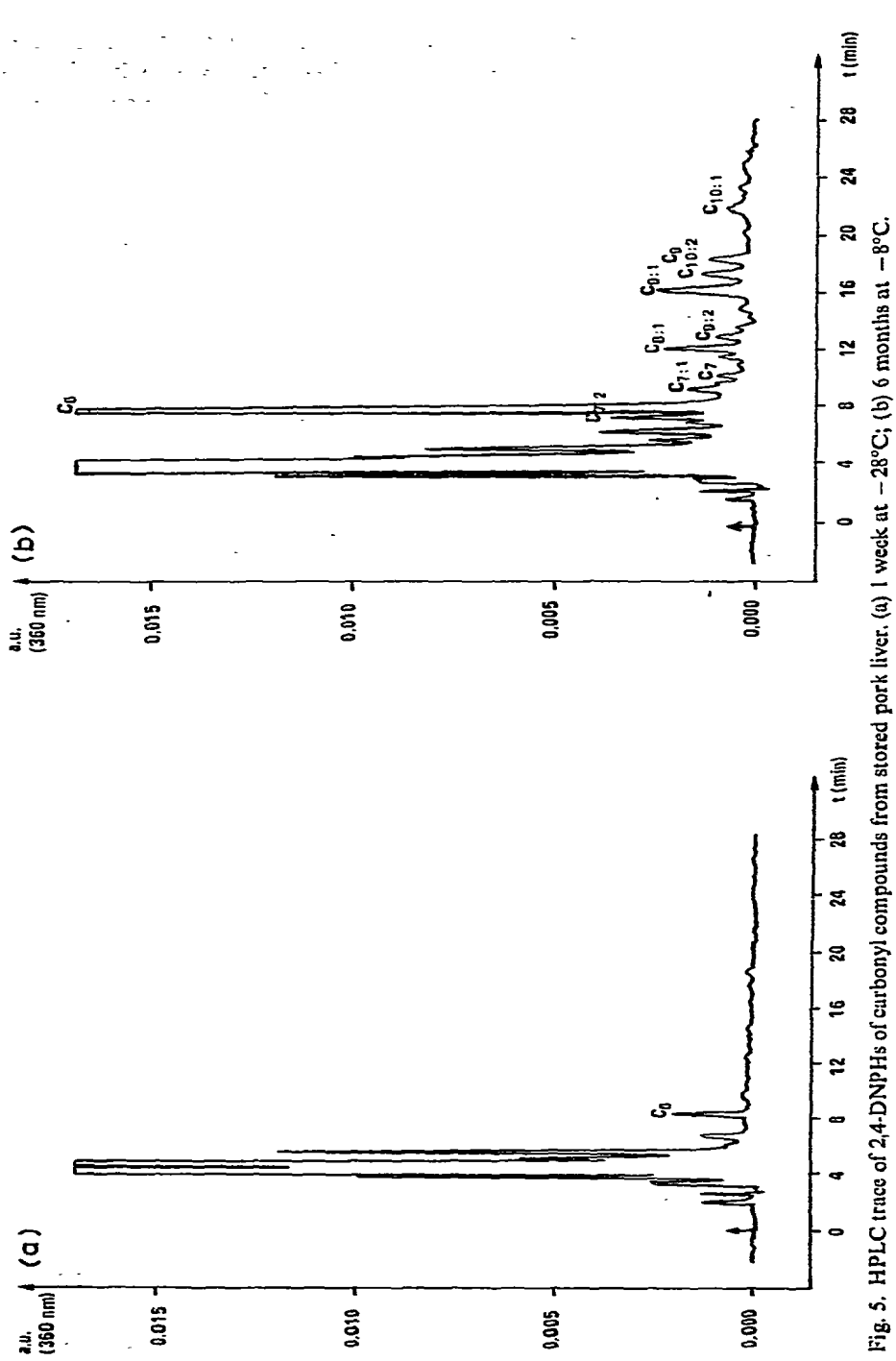

Fig. 5. HPLC trace of 2,4-DNPHs of carbonyl compounds from stored pork liver. (a) 1 week at −28°C; (b) 6 months at −8°C.

of rancidity in meat samples during storage at low temperatures. It includes an improved derivatization procedure for 2,4-dinitrophenylhydrazones which will be published elsewhere. The complete method may be of value also for carbonyl determination at trace levels in the fields of biological, medical and environmental research.

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