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ScienceDirect
Journal of Chromatography A, 1140 (2007) 131–139
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

High-temperature micro liquid chromatography for lipid molecular species analysis with evaporative light scattering detection

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Received 28 July 2006; received in revised form 15 November 2006; accepted 23 November 2006
Available online 11 December 2006

Abstract

The need for a rapid and sensitive chromatographic technique for analyzing lipid molecular species, has led to the development of an high-temperature micro liquid chromatographic system (HTLC) coupled to an evaporative light scattering detector. The increased diffusion coefficients and reduced viscosity at higher temperatures allowed lipids to be analyzed rapidly with solvents differing from those classically used in lipids chemistry. Hypercarb, a reverse phase material, was used for its different properties including heat resistance in high temperature micro HPLC. We have investigated the temperature effect on kinetic performances in HTLC, established pure solvents eluent strength at high temperature and studied different classes of lipids with seven pure solvents. We found that it was possible to use alcohols solvents in the mobile phase to elute lipids without the use of chlorinated solvents. A quick and simple method was developed to analyze a complex lipid simple, ceramide type III and type IV.

Keywords: High-temperature liquid chromatography; Micro column liquid chromatography; Evaporative light-scattering detection; Porous graphitic carbon; Lipids

1. Introduction

Lipid biology is evolving toward lipidomics [1] and high throughput methods are needed to take up this analytical challenge. Lipidomics rely heavily on the performances of LC–MS–MS which permits the profiling of closely related compounds [2–4]. Speeding up the chromatographic process without loss of efficiency would allow a valuable increase in the amount of information available from lipid samples.

The slow separation process encountered in chromatography give rise to several developments such as the use of monolithic columns [5–7], ultrahigh pressure [8,9] or high-temperature [10–12]. This last approach seems to be particularly interesting in the field of lipid molecular species profiling where nonpolar stationary phases are used in conjunction with non-aqueous solvents.

The primary consideration is solvent polarity: it has been found that increasing the temperature increases solvent strength [13,14]. Such approaches would allow extending the choice of utilizable solvents. This should avoid the use of hazardous solvents such as chlorinated solvents which are classically used in lipid analysis [15]. The second consideration is solvent viscosity and its consequences on column back pressure. Solvent viscosity decreases with increasing temperature allowing the use of high flow rates for high speed analysis [16,17]. This last point is in apparent contradiction with the former since high flow rate also means an increased solvent consumption. This drawback can be limited using micro-chromatographic techniques which also bring some significant advantages with regard to the chromatographic process [18,19]. Apart from the expected increase in sensitivity provided by reducing the column diameter, micro chromatography possesses the advantage to simplify the equipment, as fused silica tubing, which can be used at high temperature. Moreover, column hardware possesses less thermal inertia than conventional systems.

Except in the specific field of polymer separation where size exclusion chromatography is occasionally used at elevated temperatures, few studies report the use of HPLC at elevated temperatures with non-aqueous mobile phases [16,20]. A careful selection of solvent must be realized to ensure safety of operation. Risks are about the combinated effect of high temperature and pressure but also the health hazard. Form this later aspect,

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doi:10.1016/j.chroma.2006.11.067

by limiting solvent rejection, this type of chromatography could assert a move toward "green chemistry".

A recent review outlines the use of temperature programming with capillary and microbore columns [10]. High-temperature chromatography need some thermally stables stationary phases [21]. Specific thermally stable columns are used in order to prevent risks of major damage and degradation of stationary phase. Traditional silica based column packing demonstrated an acceptable stability up to 60 °C when used with aprotic solvents. Alternatives such as zirconia based stationary allow the use of temperature up to 200 °C [9,22–24]. Apart from silica or zirconia grafted stationary phases, hypercarb is a thermally resistant stationary phase with interesting characteristics with regard to lipid analysis [25].

In the present study, we focussed on the development of HTLC method with PGC with special attention to the solvents usable with this stationary phase at high temperature. In addition, as the decrease in solvent viscosity has also some important effect on diffusion coefficients, the influence of the solvent temperature on kinetic performances was assessed but also the improvement of solvents eluent strength with temperature.

The detection method used all along this study is evaporative light scattering detection (ELSD). ELSD is a widely accepted detector for lipid analysis. It processes a vast compatibility with a wide range of solvent and gradient elution [26–28]. The use of ELSD has already been reported in both HTLC [20,29] and it has proven to be usable in micro LC [30–33].

2. Experimental

2.1. HTLC-evaporative laser light scattering detection

The HTLC system consisted of a Micro Tech Scientific INC pump (Bio-Tek Kontron Instrument, Milan, Italy), a Valco manually operated injection valve equipped with a 100 nl internal loop volume (Valco Instruments, Houston, TX, USA), a Hewlett Packard 5890A gas chromatograph as the column oven. Detection was performed with a Eurosep DDL 31 (Eurosep Instruments, Cergy, France) which several adaptations for micro LC were realized by modifying the nebulizer. The detector was modified by sliding a fused-silica capillary of appropriate inner dimension (50 μm I.D., 375 μm O.D.) into the standard nebulizer nozzle. Experiments were executed at 1 bar air pressure and the nebulizer was set at 40 °C, the drift tube at 50 °C. The photomultiplier was set at 600 V, which was the recommended voltage with the 50 W halogen lamp.

The capillary column was connected between the injector and the nebulizer in the detector by a fused-silica capillary (50 μm I.D., 375 μm O.D. 50 cm length). This one acted as a restrictor, preventing the mobile phase from boiling with the increasing temperature. The mobile phase was continuously degassed with helium, for oxygen elimination, to avoid solute degradation and possible corrosion problems. The data acquisition was processed with a PC-integrator KromaSystem 3000 (Bio-Tek Kontron Instrument, Milan, Italy).

2.2. Capillary column

The stationary phase hypercarb was provided by Thermo Hypersil-Keystone (Bellfonte, PA, USA). The capillary column, 5 μm 150 × 0.53 mm, was packed in our laboratory according to Thermo Hypersil-Keystone packing procedure.

2.3. Materials and reagents

The fused-silica capillaries came from Polymicro Technologies (Phoenix, AZ, USA), unions, ferrules and nuts in stainless steel were from Valco Instruments (Houston, TX, USA). Butan-1-ol and propan-1-ol were from Merck (Darmstadt, Germany) and, methanol, propan-2-ol, ethanol, ethyl acetate, chloroform and acetonitrile were of HPLC grade from VWR International SAS (Fontenay[s]/Bois, France). All solutes used in this study were from Sigma–Aldrich (St. Quentin Fallavier, France).

Concentrations of the injected solutions were 250 μg ml^{-1} for fenuron and ceramides type III and IV and 500 μg ml^{-1} for paracetamol, squalene, ceramide IIIB, tripalmitin and cholesterol.

3. Results and discussion

3.1. Influence of high temperature on micro column efficiency

The plate number, N, which is related to the plate height, H, by the equation $N = L_{col}/H$, L_{col} being the column length, express the column efficiency. By using reduced parameters h and ν defined by $h = H/d_p$ and $\nu = ud_p/D_m$, d_p being the particle diameter and D_m the molecular diffusion coefficient of the solute in the mobile phase, the reduced plate height can be given by Knox equation [34].

$$h = A\nu^{1/3} + \frac{B}{\nu} + C\nu \quad (1)$$

The A term is related to the packing quality. The B term accounts for the longitudinal (axial) diffusion so reflects the geometry of the eluent in the column. This term becomes significant at low flow rates. The C term expresses the effect of mass transfer resistances in both mobile phase and stationary phase and this term become predominant at high flow rate.

In general, elevated temperatures are beneficial for the column efficiency. However, the three coefficients of the Knox equation are differently affected. According to Li [22], a small decrease of A with increasing temperature is observed. And according to Kephart [23], A is showed to decrease about 25% between 100 and 150 °C. However, Yan et al. [24] has found that A is relatively constant from 25 to 150 °C. At last, according to Greibrokk and Andersen [10] the effect of temperature on the A term is not very clear or even uncertain. But for B and C terms, in a general way, increasing temperature causes decreasing of C term and increasing of B term.

Most of the early studies in HTLC were carried out with conventional columns (4.6 mm I.D.) within a moderate range of temperature and with hydro organic mobile phases. In these