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ORIGINAL PAPER

**Jasmina Kožar Logar · Mateja Šikovec · Alenka Malej
Mladen Franko**

# The effects of eluent mixing on TLS detection in gradient elution HPLC

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**Abstract** The effects of changing solvent composition on the LOD of TLS detection in gradient elution HPLC have been studied from the perspective of thermo-optical properties of the solvent. Hyphenated gradient high-performance liquid chromatography (HPLC)–thermal lens spectrometry (TLS), was used to separate and detect 13 carotenoid compounds and two chlorophylls. Utilization of mixing coils into the system reduces the inhomogeneities during eluent changes and therefore enables the application of thermal lens detection in the gradient HPLC method. For gradient chromatographic conditions in which the thermo-optical properties and related enhancement factor change as much as 50% over 10 min, the LODs for the TLS detector were enhanced by as much as three times in comparison with UV-Vis detection. For the isocratic part of the chromatogram, up to a tenfold improvement of LODs was achieved with TLS detection.

**Keywords** Thermal lens spectrometry (TLS) · High-performance liquid chromatography (HPLC) · Gradient elution · Pigment · Carotenoid · Chlorophyll · Limit of detection (LOD)

**Abbreviations** *TLS* thermal lens spectrometry · *HPLC* high-performance liquid chromatography · *LOD* limit of detection · *DHI* Danish Hydraulic Institute · *UV-Vis* ultraviolet-visible range · *IC* ion chromatography · *CE* capillary electrophoresis

J.K. Logar · M. Šikovec · M. Franko (✉)
Nova Gorica Polytechnic, School of Environmental Sciences,
P.O. Box 301, 5001 Nova Gorica, Slovenia
e-mail: mladen.franko@p-ng.si

A. Malej
National Institute of Biology, Marine Biology Station,
Fornače 41, 6330 Piran, Slovenia

*Present address*:
J.K. Logar
National Institute of Biology, Marine Biology Station,
Fornače 41, 6330 Piran, Slovenia

*Present address*:
M. Šikovec
Institute Jožef Stefan, Jamova 39, 1000 Ljubljana, Slovenia

## Introduction

In thermal lens spectrometry the absorbance is measured indirectly via the so-called thermal lens effect, which was first described by Gordon et al. [1]. Optical absorption and subsequent heating of the sample in regions confined by the dimensions of the excitation laser beam changes the temperature distribution across the irradiated sample. A lens-like element is created within the sample through the temperature dependence of refractive index of the sample, which influences the propagation of the beam.

Thermal lens spectrometry is more sensitive than conventional transmission spectrometry because the photothermal effect amplifies the measured optical signal (relative change in the beam intensity) [2]. This amplification, referred to as the enhancement factor [3], is the ratio of the signal obtained using photothermal spectrometry to that of conventional transmission spectrometry. Enhancement factor depends on the thermal and optical properties of the sample and the properties of the pump and probe laser beam, which can be seen from Eq. (1):

$$\frac{\Delta I}{I} = \left(-\frac{dn}{dT}\right)\frac{P A}{\lambda k} = E A, \quad \text{where} \quad E = \left(-\frac{dn}{dT}\right)\frac{P}{\lambda k} \tag{1}$$

with $\Delta I/I$ being the relative change of the probe beam intensity, $A$ the absorbance, $E$ the enhancement factor, $dn/dT$ temperature coefficient of refractive index, $P$ power of the excitation source, $\lambda$ wavelength of the probe beam, and $k$ thermal conductivity.

Recently TLS has found many applications in chemical analysis, including its hyphenation with separation techniques such as ion chromatography (IC) [4, 5, 6, 7], capillary electrophoresis (CE) [8, 9, 10, 11], and isocratic HPLC [2, 12, 13]. Despite its great potential, TLS has not been routinely used in combination with gradient HPLC,

which provides much higher resolving power for many classes of compounds. Reasons can be found in the nature of TLS signal, which is strongly dependent on physical properties (*dn*/*dT, k*) of the chromatographic eluent, that is, the medium in which the measurement is performed. During the gradient elution, the composition of the eluent and therefore its optothermal properties are changed, which affects the TLS signal as well as the noise level of the base line.

Only one full paper [14] about measurements of pesticides and one preliminary conference proceeding [15] in which hyphenated gradient HPLC-TLS was used are known from the literature.

Higher LOD in comparison with the isocratic separation was reported due to a higher noise resulting from continuous oscillation of the baseline. The reason for these oscillations was attributed to the incomplete mixing of constituents of eluents or the release of heat caused by the mixing. However, no attempt to eliminate or reduce these unwanted effects or improvement in the performance of the HPLC-TLS technique under gradient elution conditions was mentioned in the first paper, while our preliminary study [15] has indicated that significant improvements can be made by improving the solvent mixing.

It was therefore the objective of this work to investigate the possibilities of improving the performance of TLS detection and to analyze the changes of thermo-optical parameters during the gradient elution HPLC to enable selection of chromatographic eluents and optimization of chromatographic conditions to achieve the lowest LOD.

Carotenoids were selected as model compounds for the purpose of this work since they are one of the most intensively studied groups of compounds from the point of view of HPLC–TLS determination [15, 16, 17, 18, 19], and their behavior under the isocratic HPLC conditions is well understood. To simulate the complexity of the environmental samples such as, for example, extracts of marine phytoplankton and aquatic plants, 15 compounds were used for mixed test standards in this work, and included several xantophylls and two chlorophylls. For such complex samples the gradient elution is essential.

## Experimental

### Thermal lens detection

Thermal lens spectrometric measurements were performed on a dual beam, mode mismatched TLS instrument, presented in Fig. 1. An argon ion laser (Innova 90, Coherent, 12 W), operating at 488 nm (300 mW) was used as an excitation source (pump beam). The pump beam was modulated by a mechanical chopper (Scitec Instruments) at 20 Hz and focused onto the sample cell by a 100 mm focal length lens. A helium-neon laser (model 1103P, Uniphase, 632.8 nm, 2 mW) provided the probe beam. Collinear propagation of the pump and probe beam was obtained by a dichroic mirror. The changes in the probe beam center intensity were detected with a silicon photodiode (OSD 5-E, Laser Components), which was connected to a lock-in amplifier (Model SR830 DSP, Stanford Research Systems, pre-set time constant of 300 ms) and to the computer. A 1 cm long flow-through cell (8 µL, Helma) was connected to the outlet of the chromatographic column.

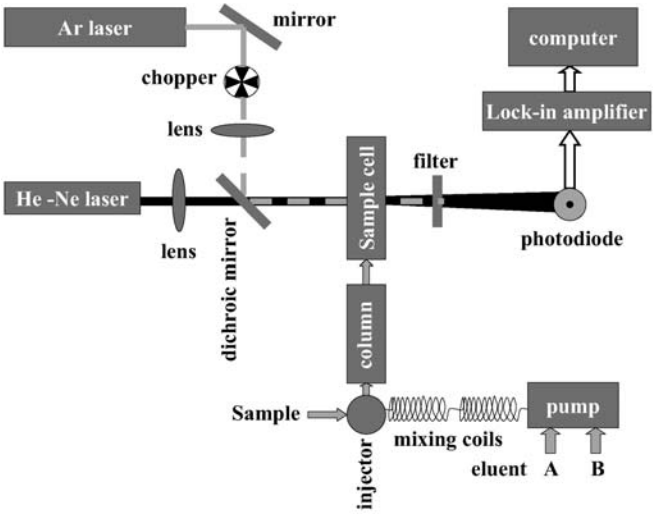


**Fig. 1** Experimental setup

### UV-Vis detection

For the comparative gradient measurements with UV-Vis detection the Hewlett-Packard HPLC system (HP 1100 series system with G1322A Degasser, G 1311A QuatPump and UV-Vis detector G1315 DAD) was used. The same measurement conditions as for the TLS detection were provided (detection at 488 nm, the same chromatographic column, eluents, samples, and temperature).

### Solvents, standards, and samples

Acetone and methanol (gradient grade, Fluka) and ammonium acetate (min. 99.0%, Merck), dissolved in distilled water, were used as eluent components.

The commercially available pigments were used as standards and are listed in Table 1. Products from DHI were diluted directly by the eluent, while other carotenoids were dissolved in tetrahydrofuran (THF, HPLC grade, Fluka). The stock solutions were prepared by adding methanol (MeOH, gradient grade, Fluka) in the ratio 3:1. Butylated hydroxytoluene (BHT, purum ≥99%, Fluka) was added (25 mg $L^{-1}$) to prevent the oxidation. The stock solutions were stored in the deep freezer at –80 °C. Working standards were prepared daily from the stock solutions by appropriate dilution with the eluent (mobile phase). The mixed standard was prepared with concentrations of single pigments as listed in Table 1. To determine the LOD values series of triplicate samples for at least four different concentrations (0.1–10.0 ng $mL^{-1}$) of a particular pigment were prepared. The measurements for each triplicate were repeated 3–6 times. The obtained correlation coefficients of calibration lines were 0.989 or better.

### Chromatographic conditions

The gradient HPLC separation was performed on a reverse phase $C_{18}$ column, 33×4.6 mm, 3 µm particle size (Pecosphere, Perkin-Elmer). In the case of TLS measurements, the column was connected to the gradient high-pressure pump (Star System 9010, Varian), equipped with a Rheodyne injector and a 20 µL injection loop. Two mixing coils (750 µL, Dionex) were inserted between the pump and the injector. The HPLC set-up and its coupling to TLS are evident from the block diagram presented in Fig. 1.

For the tested set of pigments, the gradient elution procedure with 80% methanol with 20% of 1 M ammonium acetate (eluent A) and 90% methanol with 10% acetone (eluent B) proposed by Mantoura and Llewellyn [20] and modified by Barlow et al. [21]

**Table 1** List of used pigments (13 carotenoids and two chlorophylls) and their concentrations in the mixed standard as seen in Fig. 2

| Trivial name[a] | IUPAC name | Conc. (ng mL$^{-1}$) |
|---|---|---|
| α-carotene (1) | (6'*R*)-β,ε-carotene | 5.1 |
| *trans*-β-carotene (2) | β,β-carotene | 34.3 |
| β-cryptoxanthin (4) | 3-hydroxy-β-carotene | 14.3 |
| 19'-hexano-yloxy-fucoxanthin (1) | (3*S*,5*R*,6*S*,3'*S*,5'*R*,6'*S*)-5,6-epoxy-3,3',5',19'-tetrahydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-one 3'-acetate-19'-hexanoate | 34.8 |
| alloxanthin (1) | (3*R*,3'*R*)-7,8,7',8'-tetradehydro-β,β-carotene-3,3'-diol | 35.7 |
| diadinoxanthin (1) | (3*S*,5*R*,6*S*,3'*R*)-5,6-epoxy-7',8'-didehydro-5,6-dihydro-β,β-carotene-3,3'-diol | 35.1 |
| diatoxanthin (1) | (3*R*,3'*R*)-7,8-Didehydro-β,β-carotene-3,3'-diol | 34.8 |
| fucoxanthin (1) | (3*S*,5*R*,6*S*,3'*S*,5'*R*,6'*R*)-5,6-epoxy-3,3'5'-trihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-one 3'-acetate | 34.7 |
| lutein (4) | (*3R*,3'*R*,6'*R*)-β,ε-carotene-3,3'-diol | 31.3 |
| lycopene (4) | ψ,ψ-Carotene | 23.6 |
| peridinin (1) | (3*S*,5*R*,6*S*,3'*S*,5'*R*,6'*R*)-5,6-epoxy-3,3',5'-trihydroxy-6',7'-didehydro-5,6,5',6'-tetrahydro-10',11',20'-trinor-β,β-caroten-19,11-olide 3'-acetate | 34.7 |
| Sudan I (3) | β-*apo*-8'-carotenal | 20.9 |
| zeaxanthin (4) | (3*R*,3'*R*)-β,β-Carotene-3,3'-diol | 14.3 |
| chlorophyll c2 (1) | $3^1,3^2,8^2,17^1$-hexadehydro-$13^2$-methoxycarbonyl-phytoporphyrinato-Mg(II) | 35.3 |
| chlorophyll c3 (1) | 7-demethyl-7-methoxycarbonyl chlorophyll $c_2$ | 35.0 |

[a]Suppliers of the standards are given in parenthesis: (1) DHI Water & Environment, The International Agency for $^{14}$C Determination, (2) Sigma, (3) Fluka, (4) Roth.

was chosen. It is widely used (87 times cited articles up to 2001, according to ISI citation databases) and has a relatively simple gradient procedure in terms of the number (only two) and structure (binary mixture) of eluents. The method was slightly adjusted regarding the fractional composition of eluent B (better separation of α- and β-carotene) and time protocol (prolongation because of the changed strength of eluent B). The gradient HPLC separations were started with 100% solvent A, which was changed linearly into 100% solvent B over a period of 10 min. Elution continued for 15 min using solvent B, which was afterwards changed back to solvent A. A 10 min equilibration of the column with solvent A was needed before the injection of a new sample. The chromatograms were recorded at 22 °C.

### Measurements of refractive index

The measurements of refractive index were performed on the thermostated DUR-W2 refractometer (Shmidt + Haensch GmbH&Co, version 0497) providing 0.00001 refractive index units resolution, and temperature stability of at least 0.1 °C. All eluent samples were prepared in triplicates. Measurements of refractive index were performed with 1.0 °C intervals between 17 °C and 25 °C for every triplicate. The measurements for each triplicate were repeated 5–8 times. The data were fitted to a second-order polynomial and $dn/dT$ was taken as the derivative of the polynomial at the temperature of interest [22], that is, 22 °C.

### Calculation of thermal conductivity

Thermal conductivity ($k$) for 1 M ammonium acetate was calculated according to Eq. (2) for aqueous solutions containing electrolytes, as proposed originally by Riedel [23]:

$$k(T) = \frac{\left[k_w(293\ K) + \sum \sigma_i C_i\right] k_w(T)}{k_w(293\ K)} \quad (2)$$

where $k(T)$ is the thermal conductivity of the ionic solution at temperature $T$, $k_w$(293 K) is thermal conductivity of water at 293 K, $\sigma_i$ is the coefficient characteristic for a particular electrolyte $i$ [23], and $C_i$ is the concentration of the electrolyte (mol L$^{-1}$).

The Filippov method [23] for binary mixtures was used for the calculation of thermal conductivity of the eluents as presented in Eq. (3):

$$k = w_1k_1 + w_2k_2 - 0.72w_1w_2\,(k_2 - k_1) \quad (3)$$

where $w_1$, $w_2$ are the weight fractions of components 1 and 2, and $k_1$, $k_2$ are the thermal conductivities of the pure component, respectively. The components are chosen such that $k_1 > k_2$.

## Results and discussion

The preliminary measurements by on-line coupled gradient HPLC and TLS detection have resulted in highly dispersed, unstable, and at moments irregular probe beam profiles, as observed from the probe beam projection onto a distant screen. The signal noise detected by the lock-in amplifier was generally much higher compared to measurements under isocratic HPLC conditions.

To circumvent the problem, mixing coils were implemented into the system to achieve better mixing of solvents and to reduce the eluent inhomogeneity throughout the gradient procedure. Two mixing coils (750 µL each) were inserted between the HPLC pump and the injector. This technical modification enabled TLS detection during relatively short (22 min) gradient HPLC procedures as seen in Fig. 2.

To evaluate the performance of the gradient HPLC-TLS technique with regard to the limits of detection (LOD), chromatograms of single standards were also recorded with UV-Vis detection system. The LODs were determined on

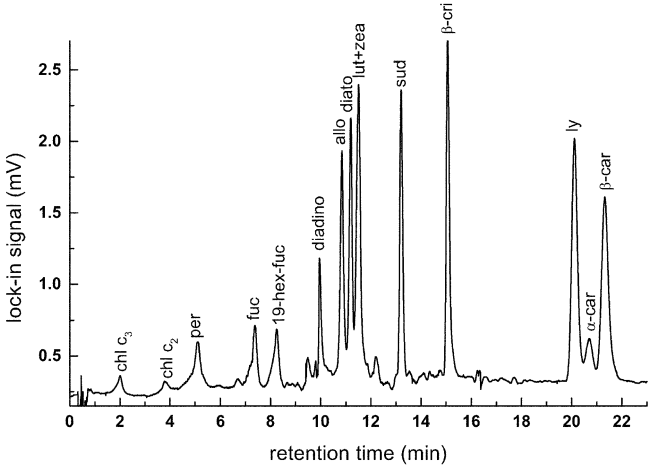


**Fig. 2** Chromatogram of 15 pigments from standard mixture of carotenoids and chlorophylls (see Table 1 for exact concentrations) obtained by using the modified gradient protocol of Mantoura and TLS detection. Legend: α α-carotene, β β-carotene, β-cri β- cryptoxanthin, 19-hex-fuc 19'-hexano-yloxy-fucoxanthin, allo alloxanthin, diadino diadinoxanthin, diato diatoxanthin, fuc fucoxanthin, lut lutein, ly lycopene, per peridinin, sud Sudan I, zea zeaxanthin, chl c2 chlorophyll c2, chl c3 chlorophyll c3

**Table 2** Limits of detection for TLS and UV-Vis detectors[a]

| Separation | Isocratic (ng mL$^{-1}$) | | Gradient (ng mL$^{-1}$) | |
|---|---|---|---|---|
| detector | TLS | UV-Vis | TLS | UV-Vis |
| chlorophyll c3 | 0.16 | | 0.32 | 2.3 |
| peridinin | | | 0.29 | 0.83 |
| 19'-hexano-yloxy-fucoxanthin | | | 0.34 | 0.74 |
| zeaxanthin | | | 0.38 | 0.50 |
| Sudan I | 0.05 | 0.40 | 0.36 | 0.51 |
| β-cryptoxanthin | 0.06 | 0.39 | | |
| lycopene | | | 0.28 | 2.9 |
| β-carotene | | | 0.24 | 1.8 |

[a]For isocratic elution of the chlorophyll c$_3$ the eluent A was used, while the isocratic measurements of Sudan I and β-cryptoxanthin were performed by eluent B.

the signal-to-noise ratio of 3 basis (signal/noise =3). The results are presented in Table 2.

Generally speaking, LODs for gradient separation with TLS detection are 1.3–10-fold lower than for the UV-Vis detection as can be deduced from Fig. 3. When compared to the previous preliminary report [15], the LOD values also show improvement (20–77%), which is mainly attributed to the longer mixing coils used in this work and to better optimization of TLS system. The new LOD values are also more reliable, since more measurement points were included in the preparation of calibration curves, particularly for lower concentrations of standards.

The smallest differences between LODs for UV-Vis and TLS detector were found for 19'-hexano-yloxy-fucoxanthin, zeaxanthin, and Sudan I, which elute within the section of chromatogram in which the solvent gradient is still in effect, or has just been completed. Therefore, the

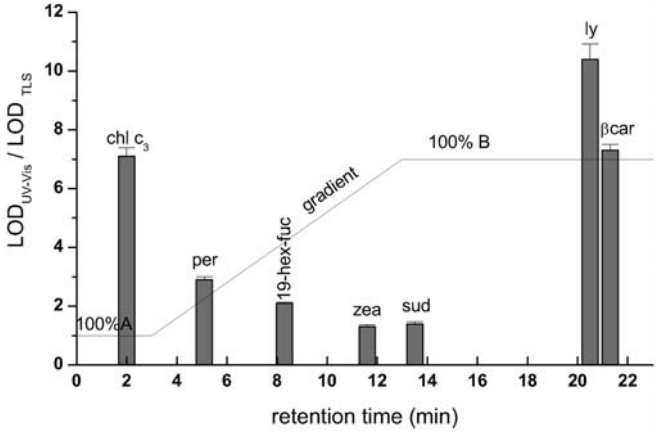


**Fig. 3** Relationship between UV-Vis/TLS limit of detection ratio and eluent composition during gradient HPLC protocol. Delay in gradient onset is due to the void volume of the mixing coils and tubing of the system

fluctuations of the baseline are as expected the highest, or have not yet been overcome by the flow of homogeneous eluent B such as in the case of Sudan I. On the other side, the differences in LOD for the two detection techniques are the largest at the beginning of the chromatogram (chl c$_3$), with UV-Vis/TLS LOD ratio of 7.2 and at the end of the chromatogram, for pigments such as β-carotene (UV-Vis/TLS LOD ratio 7.5) and lycopene (UV-Vis/TLS LOD ratio 10.4). The relationship between the UV-Vis/TLS LOD ratio and the time of elution is obvious and can be easily explained by the appearance of the mixed solvents at the detection cell, which is delayed for 3–4 min with regard to the actual beginning of the gradient mixing because of the length of mixing coils, tubing, and the column itself. This means that the chl c$_3$ elutes before the early appearance of mixed eluent in the detection cell, when the eluent is still homogeneous. Good results for lycopene and β-carotene can be explained with the argument that the two peaks are contained in a homogeneous eluent B, when the baseline has been already stabilized since the 100% eluent B begins to reach the detection cell after 13 min.

The comparison of LOD$_{TLS}$ for isocratic and gradient separation method was made for two pigments. Sudan I was chosen because of the suitable retention time; in the case of gradient elution it elutes from the column almost exactly at the time when the gradient program ends and the isocratic elution with eluent B starts. As already described, the fluctuations in signal as a result of the incomplete eluent mixing are therefore still present, but the chemical composition of the eluent is actually equal to 100% eluent B. Since the physical properties of the solvents significantly affect the LOD of the analyte through the enhancement factor, Sudan I offers the best opportunity for comparison of the methods because the results, which are presented in Table 2 as isocratic, were obtained with eluent B. Any differences in LOD can therefore be attributed directly to the local and temporal inhomogeneities in composition of the eluent. The LOD for isocratic elution

of Sudan I is 7.2 times lower than for the gradient elution with the same TLS detection.

The results for chlorophyll $c_3$ show rather similar tendency: isocratic LOD is two times lower compared to gradient elution in the case of TLS detection. On the other hand, the isocratic LOD for chlorophyll $c_3$ is three times higher than isocratic LOD for Sudan I or β-cryptoxanthin, which can be explained by the different absorption spectra and extinction coefficients of carotenoids and chlorophyll $c_3$ at 488 nm, different enhancement factors of eluents A and B, and the substantial fluorescence of chlorophyll $c_3$, which reduces the heat available for the generation of the thermal lens effect and consequently results in lower TLS signal.

Finally, the performance of TLS detection in gradient HPLC was evaluated from the point of view of changes in optothermal parameters of the eluents used during the gradient protocol. Since the *dn*/*dT* and *k* values for mixed solvents and aqueous solutions of electrolytes were not available from the literature they were measured or calculated, respectively, as described in the experimental section.

The modified Mantoura procedure was evaluated with respect to optothermal parameters of the eluents such as refractive index and its temperature coefficient, the thermal conductivity, enhancement factor, and particularly the rate of their changes during the gradient elution protocol. The change of refractive index is rather low and does not exceed 0.07% $min^{-1}$. Nevertheless, the effects of changing *n* cannot be completely disregarded, as it is an important factor involved in the observed increase of LOD in gradient HPLC-TLS, since high refractive index gradients can be formed locally between volumes of eluent with different *n*. On the other hand, evidence of considerable changes of *dn*/*dT* ($0.89\times10^{-4}$ $K^{-1}$) and *k* (0.053 W $m^{-1}$ $K^{-1}$) during the gradient chromatographic procedure were obtained from measurements and calculation. The rates of changes (how fast the optothermal properties of the eluent are changed during the gradient protocol; 2.8% $min^{-1}$ and 2.2% $min^{-1}$ for *dn*/*dT* and *k,* respectively) are even more significant than absolute differences. As shown in Figs. 4a–c these values are also much higher compared to the changes of optothermal parameters of solvents used in a previously reported HPLC-TLS procedure [14] (rates of changes being 0.5% $min^{-1}$ for *dn*/*dT* and 1.1% $min^{-1}$ for *k*).

The theoretical predictions for changes of the relative enhancement factor (Δ*E*) are shown on the Fig. 4d. They were calculated according to Eq. 1 and show good agreement with experimental data, which were tested with the baseline signal values. The differences between TLS signals at the beginning and end of the chromatograms are closely related to enhancement factors. For the modified Mantoura method, an experimental increase of 53±7% is comparable with the predicted 62% increase of enhancement factor, with possible maximum uncertainty of 16%. High deviations of predicted Δ*E* are related to the reported uncertainties of the Filippov method [23], which was used for calculation of thermal conductivity of the mixtures

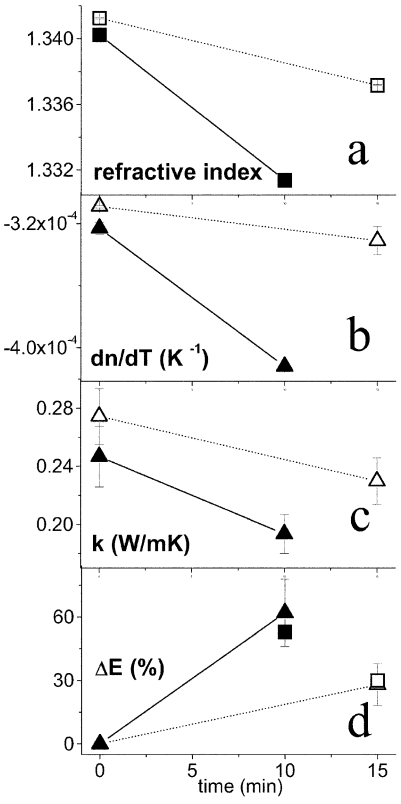


**Fig. 4a–d** Comparison of two gradient HPLC protocols in light of the refractive index *n* (**a**), temperature coefficient of refractive index *dn*/*dT* (**b**), thermal conductivity *k* (**c**), and changes of enhancement factor *E* (**d**). Legend: *black symbols* are related to the modified Mantoura method while the *white symbols* represent parameters used in reference [14]. *Triangular symbols* indicate calculated values of the parameters, while the *rectangular symbols* mean that the values were measured

that carries relative high uncertainty as seen in Fig. 4c. The thermal conductivity for eluent A in the modified Mantoura method is the most unreliable since initially the value for 1 M ammonium acetate had to be calculated according to the equation, proposed originally by Riedel [23]. A similar comparison was made for the gradient HPLC procedure used by Steinle [14]. The experimental increase of the baseline signal in the case of Steinle is 30% (read from the published chromatogram [14]) and matches excellently with predicted 29% based on changes in thermo-optical properties of the eluent. This is, however, about two times lower than the change in enhancement factor of the eluent used in the gradient protocol compared to this work. Furthermore, the change takes place over 15 min as opposed to 10 min in the modified Mantoura method. Despite larger differences and rates of change of optothermal parameters, a similar improvement in LOD compared to UV-Vis detection was achieved in both works during the gradient part of chromatographic elution (1.3–2.9 times versus 1.8–2.8 times [14] for similar beam to eluent flow orientation).

## Conclusions

The incomplete mixing of solvents in gradient HPLC combined with on-line TLS detection was confirmed as the major source of baseline noise and therefore increased LOD values. It has been demonstrated that the degree of TLS signal fluctuations is related to the changes of $dn/dT$, thermal conductivity, and the associated enhancement factor during the gradient elution protocol. Even more important are the rates of change of the mentioned parameters. By providing additional mixing of eluents through mixing coils inserted into the HPLC chromatographic system, the TLS detection is superior to conventional UV-Vis detection even for combinations of solvents where the enhancement factor changes by over 50% during 10 min gradient. It has been demonstrated that up to 15 components in a complex sample can be separated and detected with 1.3–2.9 times lower LOD during the gradient period and up to ten times lower LOD during the isocratic period of the gradient HPLC procedure when compared to conventional UV-Vis detection.

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