Thermal Lens Spectrometry in Food Analysis and Environmental Research

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The inherent high sensitivity of the TLS enables detection of various pigments such as chlorophyll or various carotenoids at sub ng/ml concentration levels. This was exploited for HPLC-TLS determination of characteristic major and minor carotenoids and chlorophylls in various species of marine phytoplankton. In this work the TLS detection was used in combination with a linear gradient HPLC for the first time. While the gradient elution provides the required selectivity of analytical procedure, the high sensitivity of TLS detection facilitates the determination of representative compounds, and adds to the accuracy of the discrimination between different phytoplankton species.
For determination of fatty acids, a novel TLS detector, which operates at CO laser wavelengths, was developed for the needs of HPLC analysis, and tested against the refractive index detector (RID). The HPLC/IR-TLS detection scheme has demonstrated sensitivity comparable to the RID detector (LOD = 0.04%) and substantial improvement in selectivity since it is capable of discriminating between fatty acids and interfering compounds like octanol, decanol and other compounds which do not absorb in the carbonyl band region.

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During the recent two years the focus in thermal lens spectrometry (TLS) has been on the application of TLS techniques in studies of processes related to food quality and environmental research. The most recent papers on analysis of foodstuffs and related samples include the utilisation of TLS for detection of various antioxidants or their precursors in fish oils[1] and in tissues like for example liver[2], as well as detection of pollutants such as organophosphate and carbamate pesticides in fruit juices[3]. In the field of environmental research a highly sensitive detection of textile dyes in water was reported[4], while determination of chromium species in water by TLS has been developed into an actually routine analytical procedure[5].

At present, the applications of TLS detection schemes are related mostly to quality, authenticity and eventual adulteration of food products where oils, fats and their constituents are among the most frequently investigated samples. Furthermore, fingerprints of natural pigments such as carotenoids can serve as an indicator of important quality parameters of vegetable oils and fruit juices. Similarly, carotenoids and chlorophylls are being determined with TLS detection and used to identify various species of marine phytoplankton and its physiological status[6].

Since the sensitivity is not always the key issue in applications listed above, the objective of this contribution is to describe some of the most recent applications of TLS in food analysis and environmental research primarily from the point of view of technique's selectivity. Improvements in selectivity of analytical procedures were achieved by replacing the isocratic HPLC (high performance liquid chromatography), which was so far engaged in TLS detection schemes, by the gradient HPLC, or by applying the IR-TLS in combination with HPLC chromatographic separation.

Gradient elution is for example essential for efficient separation of individual carotenoids and sufficient selectivity, since they all exhibit very similar absorption spectra. However, the TLS signal ($\Delta I/I$) is known to be dependent on temperature coefficient of refractive index ($\partial n/\partial T$) and thermal conductivity of the sample (k), as shown by a simplified physical description of the steady state thermal lens effect given by

$$\frac{\Delta I}{I} = \frac{2.303 AP(-\partial n/\partial T)}{\lambda k} \qquad (1)$$

where A is the absorbance of the sample, P the power of the pump laser, and λ the probe laser wavelength.

Therefore, any differences in thermooptical properties of solvents used in gradient elution would result in changing sensitivity across each chromatogram. Furthermore, uncontrollable spatial and temporal changes of eluent's density, refractive index and it's gradient due to the incomplete mixing of solvents, could disturb the propagation of the probe beam and hinder the performance of the TLS detection.

Besides the application of efficient separation techniques such as gradient elution HPLC, the required selectivity of an analytical procedure can also be introduced by spectroscopic means. This approach was exploited for the determination of fatty acids in presence of interfering compounds such as long chain alcohols, which was carried out by HPLC in combination with TLS detection in the carbonyl band region (1734 cm^{-1}) of the IR spectrum.

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Experimental

TLS spectrometers

The experiments presented in this contribution were performed on dual beam (pump/probe) thermal lens spectrometers, which were described in detail previously[7-9]. The original sample cells were replaced by the flow-through cells with 10 mm or 0.2 mm pathlength for measurements in the visible and in the IR spectral range, respectively. The laser powers provided by the Ar laser were 300 mW at 488 nm, and those provided by the CO laser were about 100 mW at 1734 cm^{-1}, as measured at the laser heads.

Samples and standards

Marine phytoplankton was collected by filtration of cultures or sea-water and carotenoids were extracted into 98 % methanol containing 0.5 M ammonium acetate at 4 °C, following sonification, centrifugation and filtration through a 0.45 µm filter. Extracts were appropriately diluted before the analysis, depending on the concentration of pigments in the sample. A mixed standard of pigments was prepared from commercially available products which were mixed in appropriate ratio to give a standard mixture containing: diadinoxanthin (c = 35.1 µg/l), peridinin (c = 34.7 µg/l), diatoxanthin (c = 34.8 µg/l), chlorophyll c2 (c = 35.3 µg/l), chlorophyll c3 (c = 35.0 µg/l), alloxanthin (c = 35.7 µg/l), 19-hex-fucoxanthin (c = 34.8 µg/l), fucoxanthin (c = 34.7 µg/l), lutein (c = 31.3 µg/l), zeaxanthin (c = 14.3 µg/l), β-cryptoxanthin (c = 14.3µg/l), α-carotene (c = 5.1 µg/l), *trans*-β-carotene (c = 34.3 µg/l), lycopene (c = 23.6 µg/l), and β-apo-8'-carotenal as internal standard (c = 20.9 µg/l).

For investigations related to the IR-TLS a commercially available (FDH, Manchester) mixture of three fatty acids containing 20 % oleic acid (C18:1, cis (n=9)), 70 % linoleic acid (C18:2, cis, cis (n=9,12)) and 10 % of conjugated linoleic acid (C18:2, cis, cis (n=9, 11)) was used as a test sample. A 99.9 % oleic acid (Merck) was used as a standard for determination of the limit of detection (LOD). Octanol, decanol and other alcohols were used as interfering compounds.

Chromatographic conditions

Chromatograms of pigments and fatty acids were recorded with 20 µl and 10 µl samples, respectively, and with 1 ml/min. eluent flow rate.

Chromatographic separations of carotenoids were performed on a Pecosphere C-18 column (33×4.6 mm) using 80 % methanol with 20 % of 1 M ammonium acetate (solvent A) and 90 % methanol with 10 % acetone (solvent B) as eluents. The gradient HPLC separations were started with 100 % solvent A, which was changed linearly into 100 % solvent B over a period of 10 minutes. Elution continued for 15 minutes using solvent B, which was afterwards changed back to solvent A. A 10 minute equilibration of the column with solvent A was needed before the injection of a new sample.

For separations of fatty acids a Vydac C-18 (250×4.6 mm) column was used with 20% chloroform in acetonitrile as eluent.

TLS detection in the case of gradient HPLC necessitated modification of conventional gradient HPLC system by addition of commercially available mixing coils for flow injection analysis in between the HPLC pump and the injector.

For comparison purposes a Stark refractive index detector was connected in series to the TLS detection system.

Results and discussion

TLS detection in gradient elution HPLC

From the experimental point of view, the main difficulty in application of TLS for detection in HPLC is to overcome problems resulting from the need of gradient elution for the HPLC determination of pigments. As it was observed during our preliminary measurements, the baseline level changed by over one order of magnitude, when solvent A was changed into solvent B. This was clearly not the consequence of the increase in TLS signal, since the enhancement factor $E=(\partial n/\partial T)/(\lambda k)$ increases by only about 20 % when solvent A is replaced by solvent B. This can be readily calculated from Eq. 1 and the thermooptical parameters of used solvents. The effect was therefore assigned to uncontrollable spatial and temporal changes of eluent's density and refractive index, which resulted from incomplete mixing of solvents. This disturbed the propagation of the probe beam, as it could be observed visually, and hindered the performance of the TLS detection.

To overcome the problem of solvent mixing in gradient elution HPLC, mixing coils (such as those used for reagent mixing in the flow injection analysis) were inserted into the HPLC system between the pump and the injector. Two mixing coils (700 µl each) were found sufficient to eliminate the effect completely and enabled recording of the first HPLC chromatograms with TLS detection in the case of linear gradient elution HPLC. The stability of the baseline and the efficiency of the separation of 15 pigments in a mixed standard can be observed on Fig 1.

Detailed inspection of baseline signal at the beginning (0 s) and at the end (1400 s) of the chromatograms reveals about 20 % increase in lock-in signal as one could expect from the difference

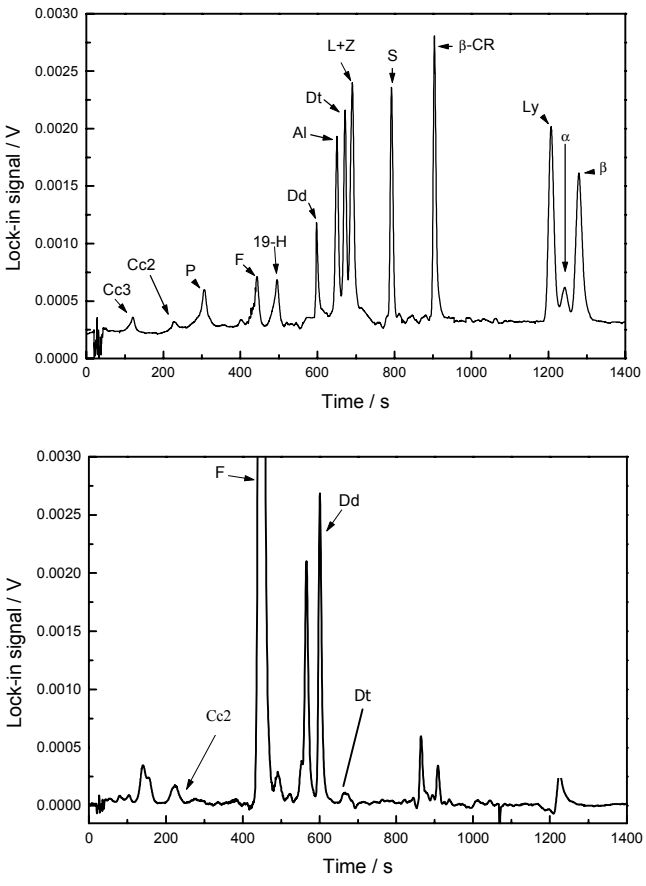

Fig. 1 Gradient HPLC chromatograms of carotenoids and chlorophylls from a standard mixture (top) and plankton species *Phaeodactylum tricornutum* (bottom). Diadinoxanthin - Dd, peridinin - P, diatoxanthin - Dt, chlorophyll c2 - Cc2, chlorophyll c3 - Cc3, alloxanthin - Al, 19-hex-fucoxanthin - 19-H, fucoxanthin - F, lutein - L, zeaxanthin - Z, β-cryptoxanthin - β-Cr, α-carotene - α, *trans*-β-carotene - β, lycopene - Ly, and β-apo-8'-carotenal - S. For concentrations of individual pigments see the experimental section (Samples and standards).

in thermooptical properties of methanol, 1M ammonium acetate, and acetone used for gradient elution. The change is however relatively small and it does not affect the interpretation of chromatograms to any considerable extent. As it can be seen from the chromatogram of pigments extracted from *Phaeodactylum tricornutum,* major components such as fucoxanthin and diadinoxanthin, can easily be identified and quantified. It is furthermore evident that, in addition to some unidentified compounds, compounds such as diatoxanthin, 19-hex-fucoxanthin (on the right hand side of fucoxanthin peak), and chlorophyll C2, are present in minor concentrations.

The limits of detection (LOD) are different for individual carotenoid compound, and were calculated to be in the range of few hundred ng/l, as presented in Table 1. The reported LOD values are slightly higher compared to previously reported LOD's for carotenoids obtained with isocratic HPLC[10], where incomplete mixing of solvents does not affect the TLS detection, since only one solvent is used.

In general, the LOD values for chlorophylls are higher in comparison to those obtained for carotenoids. This is because the excitation wavelength was not matched to the absorption maximum of chlorophylls but to carotenoids. Furthermore, high fluorescence quantum yields of chlorophylls, and the associated radiative dissipation of absorbed energy lower the magnitude of the photothermal effect.

Specific TLS detection of fatty acids in the IR

The efficiency of separation and the sensitivity of the TLS detection at carbonyl absorption band is demonstrated for a mixture of three fatty acids dissolved in the eluent on Fig. 2, where three chromatographic peaks on the TLS chromatogram can be clearly distinguished. The actual concentrations correspond to 0.2 % of conjugated linoleic acid (C18:2, cis, cis (n=9,11)), which elutes at 200 s, 1.4 % of linoleic acid (C18:2, cis, cis (n=9,12)) eluting at 215 s, and 0.4 % of oleic acid (C18:1, cis (n=9)), eluting at 245 s. Chromatograms obtained by the RID detector, connected in series with the TLS detection system, are always shown for comparison. Original chromatograms were offset in vertical direction for the purpose of clarity. The apparent horizontal offset is however due to the serial position of the RID, which requires about 15 s for the sample to reach the RID after it has been detected by the TLS.

As it can be observed on Fig. 2-A, the performances of the two detection techniques are quite similar. The RID shows about 20 % higher sensitivity, but as expected, it is at the same time less selective. This is already seen from the peak appearing at about 180 s in the RID chromatogram. The peak results from an unidentified compound, which is most probably some impurity in the linoleic acid mixture or in the solvents. The corresponding peak is however not present in the TLS chromatogram. This indicates that the compound does not contain the carbonyl group, which absorbs the CO laser radiation, and contributes to detection in the case of fatty acids. Further evidence for better selectivity of TLS detection is given by chromatograms on Fig. 2-B, which were recorded for 1 % solution of octanol in the eluent. Octanol elutes at about 210 s in the RID chromatogram and is again preceded by a small impurity peak. In the TLS chromatogram octanol produces a very weak peak which is at the limit of detection for TLS due to negligible absorbance of this compound at excitation wavelength. As a consequence, octanol, when present in concentrations up to 1 %, does not interfere with the determination of conjugated linoleic acid (C18:2, cis, cis (n=9,11)) by HPLC-TLS. However in the case of RID detection, a relatively high peak appears in the chromatogram very close to the position of conjugated linoleic acid (Fig. 2-C), and makes the determination of this compound actually impossible or subject to high systematic errors.

Similar tests were performed with other alcohols, which were also found to interfere in the determination of fatty acids,

Table 1: LOD values for selected pigments as obtained by HPLC with TLS detection

Compound	LOD/(ng/ml)
Chlorophyll C3	1.4
Peridinin	0.5
19-hex-fucoxanthin	1.0
Zeaxanthin	0.5
β-apo-8'-carotenal	0.5
β-cryptoxanthin	0.1
Lycopene	0.5
Trans-β-carotene	0.3

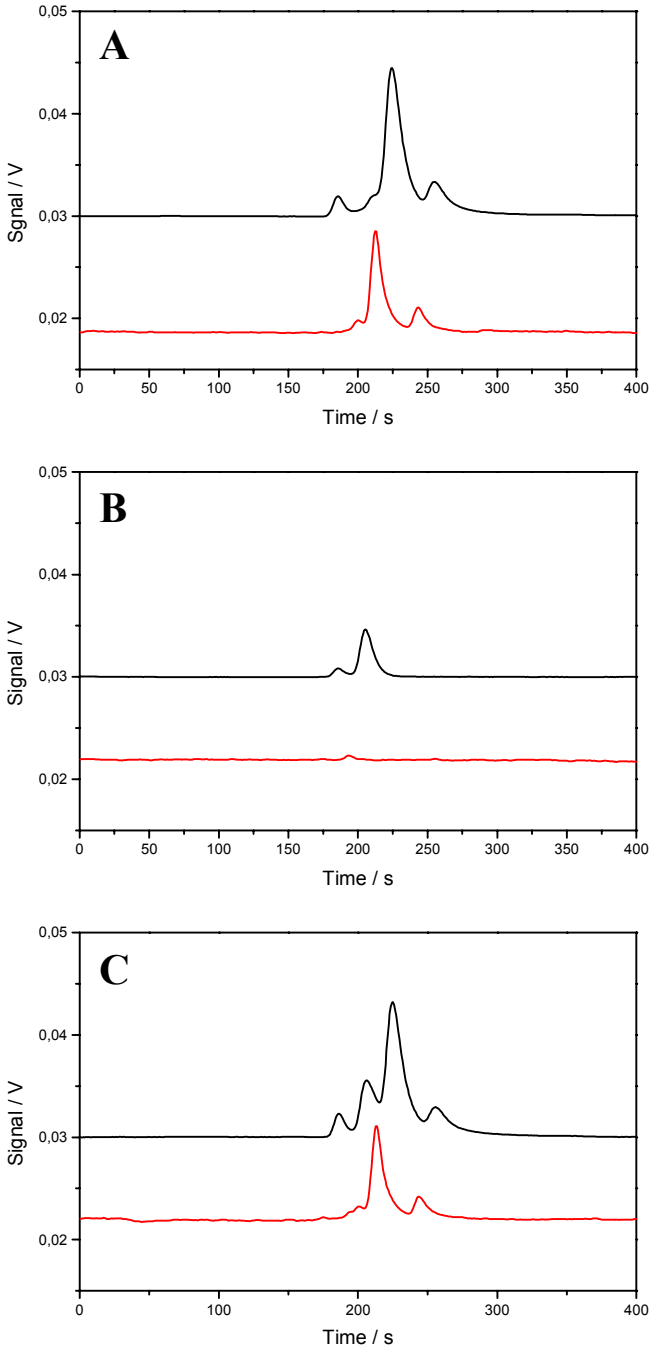

Fig. 2 HPLC chromatograms of 2% linoleic acid mix. (A), 1% octanol (B), and 2% linoleic acid mix with 1% added octanol (C). In all cases the top chromatogram is recorded with the RID detector and the bottom one with the TLS technique.

depending on their elution time. Decanol for example interferes strongly with determination of linoleic acid (C18:2, cis, cis (n=9,12)). Other longer chain alcohols appear at higher retention times and might interfere with other fatty acids present in various samples.

Experiments were also performed to establish the LOD for the newly developed HPLC-TLS method and to compare it to the HPLC-RID technique. A series of calibration curves was prepared for oleic acid as a test substance. Good linearity (R = 0.999) was found for TLS detection in the concentration range from 0.1 % to 4 % and an LOD of 0.04 % was estimated based on the signal to noise ratio of three. Due to similar absorption coefficients, the LOD values for other fatty acids are expected to be similar. From twelve repetitive HPLC-TLS determinations of 2 % oleic acid a 2.5 % standard deviation of the measurement was established and was found concentration and sample independent.

LOD's comparable to those achieved by TLS detection were also obtained for the RID detector (LOD = 0.03 % for oleic acid). However, to elucidate the actual power of TLS and RID detectors it has to be pointed out that conventional FTIR spectrometers are not able to detect fatty acids below 1 % concentration levels.

Conclusions

It has been demonstrated for the first time that despite the difficulties posed by the changing refractive index and other physical properties of solvents, the TLS technique can be used as detector in linear gradient HPLC. This opens new possibilities for application of TLS in analysis of complex samples which require highly selective analytical techniques. Examples of such applications include the determination of carotenoids in fruit juices and vegetable oils, where carotenoid fingerprints serve as indicators of authenticity or eventual adulteration and quality in general.

Furthermore, the high sensitivity of TLS detection facilitates the preparation of samples and adds to the accuracy of the method since it reduces the probability for decomposition of light and temperature sensitive carotenoid compounds. Such decomposition would results in erroneous interpretation of carotenoid fingerptints of vegetable oils and different citrus juices, as well as various freshly squeezed and thermally treated juices. The technique could also be used to observe changes in carotenoid fingerprints, which result from spoilage of the juice and can serve as an indicator of the early stages of the spoilage process.

Detection of characteristic major and minor carotenoids can also be used to identify various species of phytoplankton and it's physiologic status, which could be affected by environmental pollution and other environmental factors. For example, the highly sensitive TLS detection of pigments could be exploited in studies of cell lysis in the case of marine phytoplankton exposed to temperature shock or sudden decrease in salinity of the sea-water. Such experiments are already underway in authors lab.

At present, the LOD of gradient HPLC-TLS for determination of carotenoids and chlorophylls is still higher compared to LOD's achieved by the isocratic HPLC-TLS. This opens new possibilities for improvement of the technique's performance. Primarily this should include the development of more efficient devices for solvent mixing in gradient HPLC which would meet the needs of TLS detection and deliver homogeneous mixed solvents in terms of refractive index and density. This would reduce the baseline noise in HPLC-TLS chromatograms and could contribute to about 5 - 10 times improvement in the LOD.

An alternative for improving the selectivity of a chromatographic method is the compound specific detection, which in principle eliminates the need for a gradient HPLC that could provide the required selectivity.

By the first application of the infrared TLS in HPLC it has been demonstrated that fatty acids can be efficiently discriminated against the co-eluting compounds, which do not contain the carbonyl group. Examples of such compounds include octanol, decanol and other longer chain alcohols and represent a strong interference when nonselective detectors like the RID are used.

An LOD of 0.04 % for the HPLC-TLS determination of oleic acid at 1734 cm^{-1} CO laser wavelength was achieved. The LOD of the technique is mainly hindered by the relatively high absorption of the solvents, which also limits the choice of solvents for chromatographic separation when IR-TLS detection is used. Finding suitable solvents with low absorbance, which would also provide efficient separation on the HPLC column, is therefore essential for further progress and new applications of the infrared HPLC-TLS. Such applications could include the determination of trans fatty acids where compound specific detection in the CO_2 wavelength region would enable their determination in the presence of co-eluting cis unsaturated fatty acids.

It can be concluded that important progress has been made recently in TLS and its applications in food analysis and environmental research. With this, many new possibilities for more intensive exploitation of the TLS technique for chemical analysis in this field have been opened.

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