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Improving the sensitivity of HPLC absorption detection by cavity ring-down spectroscopy in a liquid-only cavity

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Abstract

A previously described liquid-only cavity flow cell is used to assess the feasibility of improving absorbance detection limits in liquid chromatography (LC) using cavity ring-down spectroscopy (CRDS). In this miniaturized cavity there is an optical path length of only 2 mm between the mirrors, which at the same time form the walls of the flow cell. Typical ring-down times are 65–75 ns for the eluent blank. The performance of the presented flow cell compares favorably to conventional absorbance detection: the baseline noise is determined to be 2.7×10^{-6} A.U. using averaging over 1 s. The concentration detection limits are between 15 and 20 nM (injected concentrations) for compounds with a molar extinction coefficient of 1.0–1.4 $\times 10^4$ M^{-1} cm^{-1} at the laser wavelength of 532 nm. The baseline noise as well as the absolute concentration detection limit is lowered by a factor of 30 as compared to measurements with a typical conventional absorbance detector. With an extra band broadening of only 15%, the flow cell is suitable for LC analysis.

Keywords: Cavity ring-down spectroscopy; CRDS; Liquid-only cavity; Absorption detection; Azo-dyes

1. Introduction

Improving the limits of absorbance detection for non-fluorescent analytes by liquid chromatography (LC) is a main challenge in analytical chemistry method development. Several laser-based techniques have been or currently are explored for this purpose (for a review, see Ref. [1]). Such techniques are attractive especially if miniaturization is aimed at since, contrary to lamp emission, a laser beam can easily be focussed to a very small spot without significant loss of power. It should be noted, however, that conventional absorbance detection is based upon the measurement of a small intensity difference on a large background signal, so that the sensitivity is determined by the accuracy with which $\frac{\Delta I}{I}$ can be measured. Rather than a high-intensity light source, a stable source is required. Other absorbance detection schemes, in which a signal is measured against a zero-background are being explored.

For example, degenerate four-wave mixing is based upon the production of a thermal grating in an absorbing sample using two laser beams. A third laser beam is refracted off this thermal grating, generating a fourth laser beam with an intensity depending on the absorbance of the sample. When applied as a chromatography detector [2,3], turbulence caused by the flow distorts the thermal grating, which puts limitations on this technique.

In thermo-optical absorbance measurements [4,5], absorbance of the pump beam gives rise to a temperature increase in the sample that is proportional to both the absorbance and the pump laser intensity. A second beam, which is scattered off this so-called thermal lens, is utilized to evaluate the magnitude of the absorption. A disadvantage of this technique, which has successfully been applied as a chromatography detector [6,7], is that all possible mechanical vibrations in the set-up should be eliminated.

Recently, a start was made with the implementation of cavity ring-down spectroscopy (CRDS) as an absorbance detector for LC [8,9]. While CRDS is a well-established technique for gas-phase studies [10,11], its application to the liquid phase has only recently gained interest [12–17]. In principle, CRDS offers extremely high sensitivity due to its inherent multi-pass configuration. Furthermore, while the sensitivity of conventional or the aforementioned laser-based absorption techniques is ultimately determined by the stability of the light source, CRDS is based

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on measuring the decay rate of light that is stored within a stable high-finesse cavity after abruptly terminating the excitation beam, thus higher sensitivities can be obtained. After switching off the excitation beam, the light intensity as measured behind the cavity will decay over time t according to:

$$I(t) = I(0)\exp\left[-[(1-R)+(\alpha+S)L]\frac{ct}{nL}\right] \quad (1)$$

where α denotes the absorption by the analyte, S the absorption and scatter losses introduced by the solvent (both in cm^{-1}), R the reflectivity of the mirrors, c the speed of light, L the path length in the cavity and n is the refractive index of the medium. Of course a more complex equation applies if the absorbing medium does not completely fill the cavity length, as in the design by Refs. [8,9]. Fitting the resulting decay traces to the function $I(t) = I(0)e^{-t/\tau}$ yields values for the ring-down time τ when an absorber is present in the cavity, or τ_0 for an empty cavity (i.e. blank solvent only). Losses due to the mirrors and absorption and scattering by the solvent are comprised in the latter value.

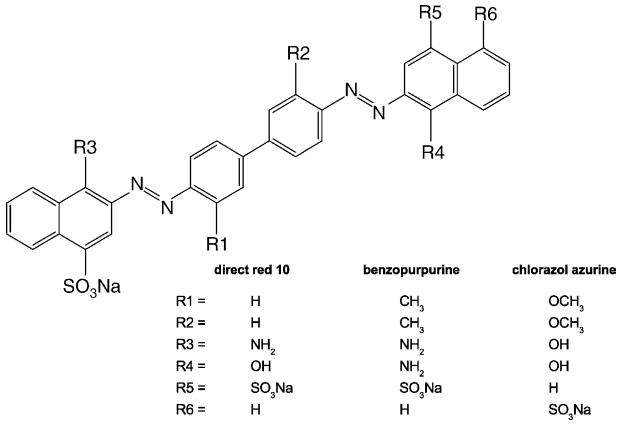
Exploratory studies indicate that CRDS is promising for detection in LC [8,9]. In these studies, a liquid flow cell that was carefully designed to approach the correct Brewster's angle at each of the four interfaces, was placed inside a cavity. Although the effective path length of the cell was only 0.3 mm, the set-up employed a 1 m cavity. This large mirror separation ensured that the decay transients were significantly longer than the laser pulses and the response time of the detection system.

As an alternative, one could develop a set-up in which there is only liquid between the mirrors. This has been explored by Hallock et al. for a cell of large dimensions [13]. In our previous study [18], the feasibility of applying this approach to a μl-sized flow cell was tested using flow injection measurements. This approach has some fundamental advantages: there are no losses due to scattering of additional surfaces in the cavity. Furthermore, since no Brewster's angles have to be considered, a large range of different eluents as well as gradient elution should be compatible with this configuration. Since τ is very short due to the small mirror separation, a repetition rate of several MHz followed by signal averaging could in principle be compatible with the proposed set-up. The disadvantage of the short decay time is that accurate determination of τ is more difficult. Short laser pulses and a fast read-out system are required, and the instrumental response time should be kept as short as possible in comparison to the decay.

This paper shows that the second approach can also be successfully implemented as an LC detector. With our liquid-only cavity which has an optical path length of 2 mm and a volume of 12 μl, we report detection limits that are significantly lower than those achieved with a conventional absorbance detector provided with a U-shaped flow cell.

2. Experimental

The performance of CRDS as a detector for LC separations was tested with a mixture of azo dyes (direct red 10, benzopurpurine, and chlorazol azurine, all obtained from Sigma–Aldrich)



	direct red 10	benzopurpurine	chlorazol azurine
R1 =	H	CH_3	OCH_3
R2 =	H	CH_3	OCH_3
R3 =	NH_2	NH_2	OH
R4 =	OH	NH_2	OH
R5 =	SO_3Na	SO_3Na	H
R6 =	H	H	SO_3Na

Fig. 1. Structures of the dyes in the test mixture: direct red 10 (FW = 697.66 g/mol), benzopurpurine (FW = 724.73 g/mol) and chlorazol azurine (FW = 758.70 g/mol).

of which the structures are shown in Fig. 1 and the absorption spectra in Fig. 2. All three have an extinction coefficient in the range of 1.0–1.4 × 10^4 M^{-1} cm^{-1} at the laser wavelength of 532 nm, no fluorescence was observed for these compounds. The LC separation was carried out isocratically, the eluent was 50% (v/v) 10 mM potassium phosphate buffer (pH 7.4) in HPLC-grade methanol. The flow rate was set to 0.8 ml/min with an Applied Biosystems 400 solvent delivery system; 50 μl of sample was injected using a six-port injection valve. The column was a Chromsep Microspher (Varian) C_{18} 100 mm × 4.6 mm (length × internal diameter) reversed phase column equipped with a guard column. For comparison, the same separation has been performed using a conventional UV–vis absorbance detector (Separations, Applied Biosystems 759a, 8 μl U-shaped flow cell with 8 mm optical path length) that was set to 532 nm. This wavelength, quite appropriate for detection of the dyes concerned (see Fig. 2), was also used in the CRDS measurements. The CRDS set-up was similar to the one described in our previous study focusing on flow-injection [18] and is schematically depicted in Fig. 3. Mirrors ($R \geq 99.996\%$ at 532 nm, 50 mm

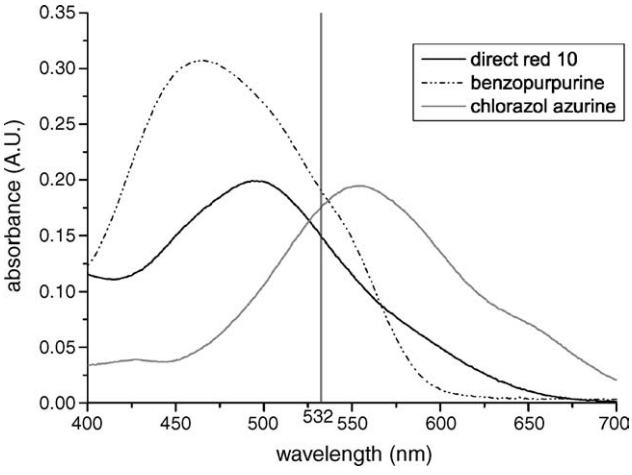


Fig. 2. Absorption spectra of the separate dyes. The concentration of the dyes is 10 ppm in 100 mM aqueous potassium phosphate buffer, pH 7.4.

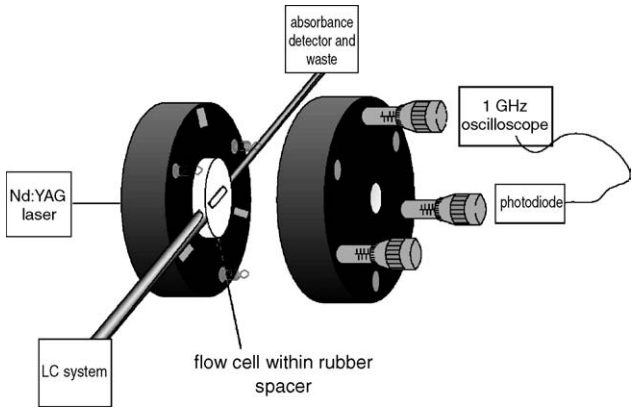


Fig. 3. Schematic diagram of the flow cell (not to scale) and the set-up. The outer boundaries of the cavity ring-down flow cell are formed by the concave mirrors, pressed leak-tight to the sides of a silicon rubber spacer. Flow is introduced via capillary tubing inserted in the spacer.

radius of curvature) were obtained from REO Inc. (Boulder, CO). In order to create the 12 μl flow cell, a 2 mm thick silicon rubber spacer with a near-elliptical hole was pressed leak-tight between the two mirrors. Flow was introduced via capillary tubing inserted in the spacer. The mirrors were in direct contact with the liquid flow. Although no noticeable degradation of the mirror quality during a day's work was observed, the mirrors needed cleaning with methanol at the end of each day and were stored in a desiccator overnight.

A Quanta-Ray Nd:YAG laser (Spectra-Physics) with the possibility of injection seeding, a repetition rate of 10 Hz and a pulse duration of 5 ns was operated at 532 nm. The transients were registered by a fast oscilloscope (Tektronix 5104 1 GHz) at a maximum sampling rate of 5 Gs/s, so that 2000 data points were available on a 400 ns time trace (typically corresponding to 5τ). Detection of the optical transient behind the cavity was done with a 4 GHz bandwidth photodiode (PHD400, Becker and Hickl) together with a 35 dB 1.8 GHz amplifier (Becker and Hickl). An auxiliary photodiode was employed to trigger the detection system. Laser light of 1064 nm was rejected using a 532 nm band-pass filter.

The response function of the detection system was determined to be about 6 ns FWHM, therefore fitting of the decays was started 10 ns after triggering. Measurements were started 20 ns or 100 data points before triggering, and the offset was determined from the measured baseline. After subtracting the baseline, decay times (typically between 65 and 75 ns in eluent flow) were fitted on-line using a Levenberg–Marquardt nonlinear least-squares fitting routine to yield a chromatogram expressed in τ.

3. Results and discussion

3.1. Optimization of excitation source

The choice of a laser system differing from the one employed previously (a mode-locked and Q-switched Nd:YAG laser with pulses of 100 ps duration at 10 Hz [18]) warrants some further remarks. Since the presently used mirrors are of higher quality, the transients are longer and other, more convenient, laser sources with long pulse duration may also be utilized. Ring-down times in excess of 50 ns allow for response times of 5 ns without loss of sensitivity.

Firstly, a Coherent Infinity single-mode Nd:YAG laser, producing pulses of 2.4 ns duration and a variable repetition rate between 10 and 100 Hz, was implemented. This system delivered narrow-bandwidth laser pulses near the Fourier transform limit of 0.014 cm^{-1}. Secondly, a Spectra-Physics Quanta-Ray Nd:YAG laser was employed, equipped with an injection-seeder ensuring single-mode operation (generating a band-width of less than 0.01 cm^{-1}); in the non-seeding mode of operation a larger bandwidth of $\sim$1 cm^{-1} was obtained. In both cases the pulse duration was 5 ns.

The long coherence lengths, associated with the narrow bandwidths, caused strong interference effects in the high-finesse cavity, resulting in large pulse-to-pulse transmission variations (over orders of magnitude) combined with periodically changing ring-down times (between 40 and 100 ns). These phenomena are related to coincidental matching of the laser mode to the cavity length L at the instant of the laser pulse, where L sensitively depends on vibrations and temperature drift of the set-up. Under such conditions the common description of CRD, in terms of non-interfering multi-passing optical trajectories in a resonator exhibiting a "white", i.e. frequency-independent response [19], breaks down. The approach of a white cavity is applicable at short coherence lengths and under excitation of a non-confocal cavity, where many transversal modes tend to fill in the transmission spectrum, as was experimentally demonstrated by [20]. One of the features of non-interference white-cavity operation of a CRDS set-up is that the transmission of the resonator equals $1 - R$, so that only a minute fraction of the incident light intensity reaches the detector.

The observed features can also be understood from a frequency-domain perspective. The free-spectral-range (FSR = $c/2L$) of our cavity of length L = 2 mm is 75 GHz, corresponding to 2.5 cm^{-1}. This mode-spacing is large with respect to the aforementioned laser bandwidths; hence, the single-mode laser frequency may or may not be in resonance with the cavity. However, when the injection-seeding in the latter Nd:YAG laser is blocked, the bandwidth is increased to $\sim$1 cm^{-1} and over a 1000 laser modes are supported.

At mirror reflectivities R of 99.996% the finesse F of the cavity, equalling $F = \pi\sqrt{R}/(1-R) = \mathrm{FSR}/\delta\nu$ is very high ($F \approx 80{,}000$) giving a modewidth of $\delta\nu = 3.2 \times 10^{-5}$ cm^{-1}. However, with a radius-of-curvature of 5 cm and L = 2 mm the cavity is sufficiently far from confocal, so that many transversal modes will be excited. Under conditions of the 1000 laser modes of the unseeded Nd:YAG laser exciting the high number of transversal modes of the miniature non-confocal resonator the condition of a white resonator, described by Naus et al. [19], was approached. Hence the unseeded Nd:YAG laser produced the stable CRDS detection conditions that were employed in the present study. Miniature CRDS-setups, like the present one, are not easily operated with single-mode Nd:YAG lasers.

3.2. LC-CRDS

In order to establish the performance of CRDS as an absorbance detector, LC was carried out for a test mixture containing the three dyes shown in Fig. 1. For further processing, the CRDS chromatograms expressed in τ were converted to $1/\tau$ and a 10 points moving average was applied. This averaging over 1 s corresponds to the time constant of an RC-circuit that is applied for electronic filtering in the conventional absorbance detector used in the comparison. A baseline ($1/\tau_0$) was fitted through the data and subtracted. The concentration of absorbing species in the cavity follows from:

$$\varepsilon C = \frac{n}{2.303c}\left[\frac{1}{\tau} - \frac{1}{\tau_0}\right] \quad (2)$$

wherein ε is the molar extinction coefficient at 532 nm and C the concentration of the absorbing species in the cavity. The resulting chromatograms could directly be converted to absorbance units (A.U.). To obtain the peak areas, Gaussian peaks were fitted through the peaks in the chromatograms.

A direct comparison between conventional absorbance detection and CRDS is shown in Fig. 4; a chromatogram was recorded using both detection systems in series. The eluent was first led into the CRDS flow cell and subsequently through the absorbance detector. Note the difference in peak heights between CRDS detection and our conventional absorbance detection in Fig. 4: since the path length is a factor of four lower for the CRDS flow cell, the peak height is also four times lower. The absence of photodecomposition of the dyes was demonstrated by reproducing the chromatographic separation with the laser turned off (not shown). Absorbance detection measurements with and without the CRDS flow cell in series using higher concentrations showed that the extra band broadening caused by the CRDS flow cell for the first eluting peak was less than 15% so that the chromatographic resolution was almost fully conserved.

The detection limit of LC with CRDS detection was determined to be 15–20 nM (injected concentration) for the three azo dyes under study at 1 s averaging time. Since the absorbance detector showed a detection limit of at least 0.50 μM, the improvement of the detection limit was about a factor of 30. The peak-to-peak noise in the LC-CRDS chromatogram was only 2.7×10^{-6} A.U. at 1 s averaging; much better than the conventional absorbance detector described in this study and comparable with the best instruments on the market [9,21].

Previously, the feasibility of using the proposed liquid-only cavity as an absorbance detector was tested using flow-injection analysis [18]. With a compound having $\varepsilon = 5.45 \times 10^4\ \mathrm{M^{-1}\,cm^{-1}}$, detection limits of 2.5 nM were obtained. At low concentrations the measurements were hindered by a background signal: upon injection of a blank, a small but measurable peak was observed. Now, in on-line LC this blank peak appears at the chromatographic dead time, removed from the peaks of interest. However, this advantage is outweighed by the dilution inherent to LC separations.

Calibration curves of the dyes (see Fig. 5) show that LC-CRDS is linear over a concentration range of 12.5–300 ppb, the R^2 values being 0.9985 or better. The error bars also illustrate a satisfactory repeatability of the CRDS peak areas and the slopes of the calibration curves are directly proportional to molar extinctions of the analytes. As regards the upper limit, at 300 ppb the ring-down lifetime τ as measured at the maximum of a peak has decreased to 15 ns, and higher concentrations are

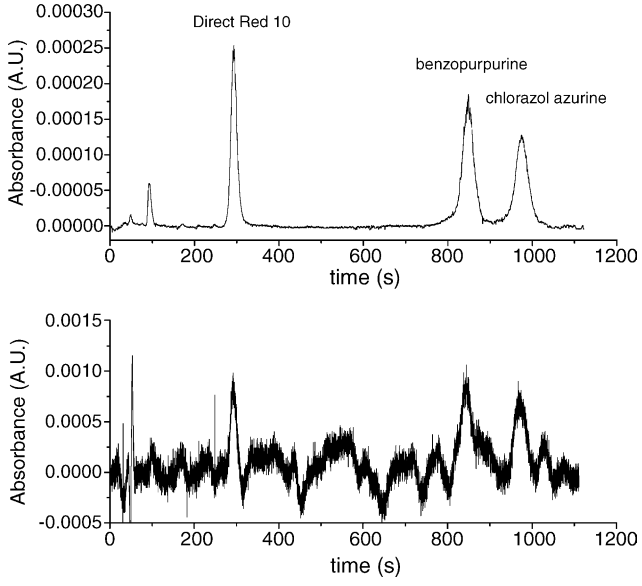


Fig. 4. Chromatogram of a mixture containing direct red 10, benzopurpurine and chlorazol azurine as detected consecutively with CRDS (top) and a conventional absorbance detector (bottom), that were connected in series. The injected concentration of each of the dyes was 300 ppb.

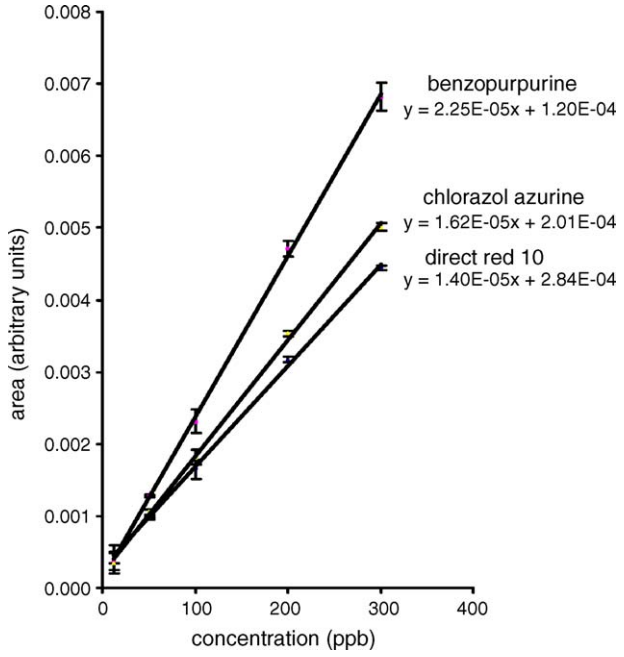


Fig. 5. Calibration curves of direct red 10 ($\varepsilon_{532} = 1.0 \times 10^4\ \mathrm{M^{-1}\,cm^{-1}}$), benzopurpurine ($\varepsilon_{532} = 1.4 \times 10^4\ \mathrm{M^{-1}\,cm^{-1}}$) and chlorazol azurine ($\varepsilon_{532} = 1.3 \times 10^4\ \mathrm{M^{-1}\,cm^{-1}}$) as measured with CRDS detection shows a linear dynamic range up to at least 300 ppb. Each data point represents an average of three measurements, with the error bar showing the standard deviation.

likely to cause problems with the fitting of the transients due to the width of the instrumental response function. Since conventional absorbance detection is feasible at such concentrations, the limited dynamic range of the CRDS detection is not a serious drawback of the technique.

4. Conclusion

The liquid-only CRDS flow cell studied here has shown a good detection performance in conventional-size LC. While its contribution to the chromatographic band broadening is only minor, excellent concentration detection limits (15–20 nM injected) were obtained for the dyes concerned. The short ring-down times resulting from the 2 mm liquid-only cavity could still be quantified with sufficient precision for analyte concentrations up to the 300 ppb level; the peak-to-peak baseline noise corresponded to 2.7×10^{-6} A.U.

These results are fully comparable with the alternative approach of Refs. [8] and [9], in which a 0.3 mm path length flow cell inside a 1 m cavity was utilized. The main advantage of our liquid-only CRDS flow cell is that it is in principle applicable for all eluent compositions and also in gradient LC since no Brewster's angles need to be considered.

Further improvements of liquid-only CRDS will be achievable by utilizing a Z-shaped flow cell, which is currently under construction. Furthermore, the decay times dealt with in the present set-up are shorter than 100 ns. This will allow the use of MHz repetition rates, implying more efficient signal averaging. Future challenges will be to reduce the cell volume to make the system compatible with micro-LC and the use of shorter laser wavelengths to broaden the applicability range of LC-CRDS.

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References

[1] T. de Beer, N.H. Velthorst, U.A.T. Brinkman, C. Gooijer, Laser-based non-fluorescence detection techniques for liquid separation systems, J. Chromatogr. A 971 (2002) 1–35.
[2] K. Fujiwara, K. Kurokawa, H. Uchiki, T. Kobayashi, Spectrosc. Lett. 20 (1987) 633.
[3] T. de Beer, G.P. Hoornweg, G.J. Grootendorst, N.H. Velthorst, C. Gooijer, Forward-scattering degenerate four-wave mixing as a potential laser-based absorption detection method in liquid separation systems: coupling to conventional-size liquid chromatography, Anal. Chim. Acta 330 (1996) 189–197.
[4] T.G. Nolan, W.A. Weimer, N.J. Dovichi, Laser-induced photothermal refraction for small volume absorbance determination, Anal. Chem. 56 (1984) 1704–1707.
[5] D.J. Bornhop, N.J. Dovichi, Simultaneous laser-based refractive index and absorption determinations within micrometer diameter capillary tubes, Anal. Chem. 59 (1987) 1632–1636.
[6] J.M. Saz, B. Krattinger, A.E. Bruno, J.C. Diez-Masa, H.M. Widmer, Thermo-optical absorbance detection of native proteins separated by capillary electrophoresis in 10 micrometer i.d. tubes, J. Chromatogr. A 699 (1995) 315–322.
[7] M. Qi, X.-F. Li, C. Stathakis, N.J. Dovichi, Capillary electrochromatography with thermo-optical absorbance detection for the analysis of phenylthiohydantoin-amino acids, J. Chromatogr. A 853 (1999) 131–140.
[8] K.L. Snyder, R.N. Zare, Cavity ring-down spectroscopy as a detector for liquid chromatography, Anal. Chem. 75 (2003) 3086–3091.
[9] K.L. Bechtel, R.N. Zare, A.A. Kachanov, S.S. Sanders, B.A. Paldus, Moving beyond traditional UV–visible absorption detection: cavity ring-down spectroscopy for HPLC, Anal. Chem. 77 (2005) 1177–1182.
[10] M.D. Wheeler, S.M. Newman, A.J. Orr-Ewing, M.N.R. Ahsfold, Cavity ring-down spectroscopy, Faraday Trans. 94 (1998) 337–351.
[11] G. Berden, R. Peeters, G. Meijer, Cavity ring-down spectroscopy: experimental schemes and applications, Int. Rev. Phys. Chem. 19 (2000) 565–607.
[12] S. Xu, G. Sha, J. Xie, Cavity ring-down spectroscopy in the liquid phase, Rev. Sci. Instrum. 73 (2002) 255–258.
[13] A.J. Hallock, E.S.F. Berman, R.N. Zare, Direct monitoring of absorption in solution by cavity ring-down spectroscopy, Anal. Chem. (2002) 1741–1743.
[14] A.J. Hallock, E.S.F. Berman, R.N. Zare, Ultratrace kinetic measurements of the reduction of methylene blue, J. Am. Chem. Soc. 125 (2003) 1158–1159.
[15] A.J. Hallock, E.S.F. Berman, R.N. Zare, Use of broadband, continuous-wave diode lasers in cavity ring-down spectroscopy for liquid samples, Spec. Tech. 57 (2003) 571–573.
[16] A.J. Alexander, Reaction kinetics of nitrate radicals with terpenes in solution studied by cavity ring-down spectroscopy, Chem. Phys. Lett. 393 (2004) 138–142.
[17] Z. Tong, A. Wright, T. McCormick, R. Li, R. Oleschuk, H.-P. Loock, Phase-shift fiber-loop ring-down spectroscopy, Anal. Chem. 76 (2004) 6594–6599.
[18] B. Bahnev, L. van der Sneppen, A.E. Wiskerke, F. Ariese, C. Gooijer, W. Ubachs, Miniaturized cavity ring-down detection in a liquid flow-cell, Anal. Chem. 77 (2005) 1188–1191.
[19] H. Naus, I.H.M. van Stokkum, W. Hogervorst, W. Ubachs, Quantitative analysis of decay transients applied to a multimode pulsed cavity ringdown experiment, Appl. Opt. 40 (2001) 4416–4426.
[20] G. Meijer, M. Boogaarts, R. Jongma, D. Parker, Coherent cavity ring down spectroscopy, Chem. Phys. Lett. 217 (1994) 112–116.
[21] JMSTsystems, http://www.jmstsystems.-com/docs/vuv-30rev4_2.pdf.