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# Absorbance detection of amino acids by laser wave mixing in microbore liquid chromatography

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**Abstract**

Nonlinear optical phase conjugation by degenerate four-wave mixing is demonstrated as a sensitive ''absorbance'' detection method for microbore high-performance liquid chromatography. An argon ion laser operating at the 488-nm line is used as the excitation light source to generate the wave-mixing signal for dabsyl-labeled amino acids. Advantages of the nonlinear laser detection method include: virtually 100% optical signal collection efficiency, generation of the signal in the form of a coherent laser beam, signal measurement against a virtually dark background, reliable detection of small absorbance values, excellent detection sensitivity for both fluorescing and non-fluorescing analytes, relatively simple one-color one-laser optical setup, and low power or energy requirements for continuous-wave or pulsed lasers. Using our one-laser one-color nonlinear laser detector for ''absorbance'' measurements in liquid chromatography, we report a crude preliminary ''injected'' detection limit of 780 fmol for glycine. © 1998 Elsevier Science B.V.

*Keywords:* Detection, LC; Laser wave mixing detection; Degenerate four-wave mixing detection; Amino acids

## 1. Introduction

Microbore high-performance liquid chromatography (HPLC) offers many desirable features including high resolution, low solvent consumption, small detector volume and excellent mass detection sensitivity [1]. When conventional absorbance or fluorescence optical detection methods are used in conjunction with narrow-bore columns, many problems including short optical path lengths, large amounts of stray radiation and poor light throughput, can seriously limit the performance of a conventional optical detector. However, when a laser is employed as the excitation source, some of the problems are less significant since a laser beam can be focused into a very small spot to obtain extremely small detection probe volumes. Several sensitive low-volume laser-based detectors have been developed to meet the requirements of microbore chromatography, including laser-induced fluorescence detectors [2,3] and thermo-optical detectors [4–6] that offer significant improvements in sensitivity and detection limits over conventional nonlaser methods. However, these laser-based methods have some limitations. For example, laser-induced fluorescence detectors are effective only to detect reasonably fluorescing analytes. In a thermal-lens detection method, divergence of a probe laser beam is monitored as an intensity loss at the center of the beam due to formation of a thermo-optical lens in the sample. Hence, the thermal-lens signal is based on the measurement of the small change or difference of a strong probe intensi-

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ty, rather than a strong positive signal monitored against a zero background as in degenerate four-wave mixing (D4WM) methods. In addition, most of the thermal-lens detection methods require two lasers, one as the pump beam and the other as the probe beam.

In this report, we present the application of D4WM as a one-color one-laser detector for microbore HPLC. An argon ion laser operating at the 488-nm line is used as the excitation light source. Amino acids derivatized with dabsyl (4-dimethylaminoazobenzene-4′-sulfonyl chloride) are detected using the D4WM detection method. Dabsyl chloride is a non-fluorescing compound with a high molar absorptivity ($3 \cdot 10^4\ M^{-1}\ cm^{-1}$) near the 488-nm line of an argon ion laser. Dabsyl amino acids are relatively easy to prepare, and are stable under photon illumination [7]. Chromatographic separation and detection of amino acids at trace concentrations are of interest to a number of biomedical fields [8,9]. In our experiment, four dabsyl amino acids are isocratically separated with methanol–acetate (60:40) buffer at pH 5 on a reversed-phase microbore (1.0 mm I.D.) column.

D4WM is a versatile laser technique increasingly used in optics and image processing fields, such as lensless imaging, real-time holography and for characterization of materials, such as third-order nonlinearity measurements [10–12]. In a D4WM experimental setup, a forward pump beam $E_f$, a backward pump beam $E_b$, and a probe beam $E_p$, are mixed inside a nonlinear medium (i.e., the analyte). The fourth beam (i.e., the phase conjugate signal beam) $E_s$ originates inside the analyte nonlinear medium and retraces the probe beam path. Since the D4WM signal is a coherent laser beam, it can be collected and detected very conveniently with excellent optical collection efficiency.

As a novel nonlinear laser spectroscopic technique, D4WM offers many advantages including high spectral resolution, excellent detection sensitivity and ease of signal isolation from all optical background noise. We reported effective applications of D4WM as a sub-Doppler high-resolution spectroscopic method using hollow-cathode discharge atomizers [13–15] and analytical flames [16–19]. In addition, we demonstrated ultrasensitive attomole-level detection limits for D4WM detectors using liquid flow cells [20,21].

Unlike laser-based fluorescence detection methods, D4WM could detect both fluorescing and non-fluorescing analytes with excellent (e.g., attomole level) detection sensitivity [20]. Since the D4WM signal beam is a time- and space-reversed replica of the probe beam, it is a coherent laser beam, and hence, virtually 100% of the signal could be sent to a photodetector. Unlike many thermal-lens or thermal-optical spatial-grating-based detection methods, D4WM requires only a single-laser setup instead of two-color two-laser setups. We have demonstrated D4WM as a sensitive detection method using low energy (nJ range) pulsed lasers and simple low power (mW range) continuous-wave lasers. Since the input laser beams can be mixed conveniently inside a small probe volume, this nonlinear detection method is suitable for on-column detection of capillary columns in various chromatography and electrophoresis systems.

## 2. Experimental

The D4WM experimental setup used in this report is similar to that described in our previous report [20] except that a liquid chromatograph is added as shown in Fig. 1. An argon ion laser (Coherent, Palo Alto, CA, USA; Model Innova 90-6) operating at 488 nm is used as the excitation light source. The laser beam polarization is purified by a polarizer and then the beam is split by a 70/30 beam splitter. The reflected beam from the first beam splitter is further

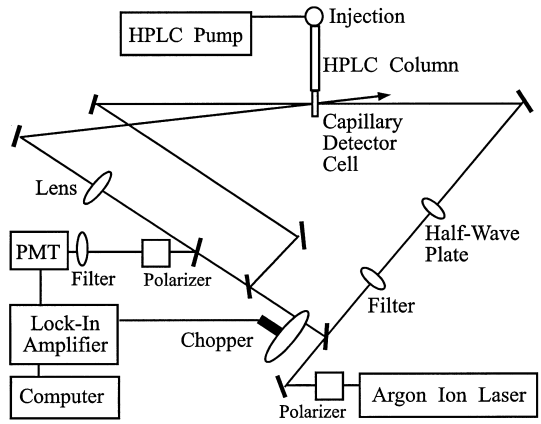


Fig. 1. Experimental arrangement for laser degenerate four-wave mixing LC detector.

split by a second 50/50 beam splitter. The reflected beam from the second beam splitter is used as the forward pump beam, and the transmitted beam is used as the probe beam. The transmitted beam from the first beam splitter is used as the backward pump beam. A half-wave plate is used to rotate the polarization plane of the backward pump beam by 90° so that the returning forward and backward pump beams can be rejected by the polarizer, and hence preventing them from entering the argon ion laser cavity. The backward pump beam is attenuated by the use of a 0.4-optical-density neutral density filter. The input beams are focused to obtain a small laser probe volume inside the capillary detector cell. The D4WM signal generated by the detector cell is conveniently extracted by a 30/70 beam splitter and then sent to a photomultiplier tube (PMT; Hamamatsu, Middlesex, NJ, USA; Model R928). Since the phase conjugate signal beam has the same polarization plane as that of the backward pump beam, background scattering noise caused by the forward pump and probe beams can be rejected by placing a cross-polarized polarizer. A 488-nm laser-line filter is used in front of the PMT to further minimize background noise. The output of the PMT is passed through a current-to-voltage converter and sent to a lock-in amplifier (Princeton Applied Research, Princeton, NJ, USA; Model 5207). A mechanical chopper is used to modulate the amplitude of both the forward pump beam and the probe beam at 30 Hz. The output voltage of the lock-in amplifier is recorded by a strip chart recorder and a personal computer.

A 0.2-mm square-bore capillary tube glued inside a 0.38 mm I.D. poly(vinyl chloride) tubing is used as the D4WM detector cell. The two pump beams are aligned so that they are exactly counter propagating, first without the cell in the optical path. The cell is then inserted in the pump beam path and the probe beam is aligned to intersect the pump beam inside the cell at a small angle (e.g., less than 0.5°). The optical alignment is then optimized by maximizing the intensity of the D4WM signal beam that is visible to the naked eye using a highly concentrated (i.e., $\sim 5 \cdot 10^{-6}$ *M*) test solution of eosin B in ethanol. The analyte solution is pumped at a flow-rate of 0.01 ml/min by a peristaltic pump (Rainin, Woburn, MA, USA).

The liquid chromatographic system (Pharmacia) consists of a pump (Pharmacia, Model 2248) with a flow-rate range from 0.01 ml/min to 10 ml/min, a pump controller (Pharmacia, Model 2252) and a solvent conditioner (Pharmacia, Model 2156). The injector system (Rheodyne, Model 7125) has a 5-μl sample loop. A 25 cm×1.0 mm I.D. microbore column (Spherisorb ODS2, Phase Separations, Norwalk, CT, USA) is used to test our D4WM detector. The mobile phase, a 60:40 mixture of methanol and buffer (0.05 *M*, acetate pH 5), is filtered through a 0.22-μm filter at a flow-rate of 0.01 ml/min. The detector cell is a 0.2-mm square-bore capillary tube (Wale Apparatus, Hellertown, PA, USA) that is epoxied inside a piece of 1/100 in. I.D.×1/16 in. O.D. stainless steel tubing, which is in turn connected to the end of the microbore column (1 in.= 2.54 cm). The laser beams pass through the capillary detector cell approximately 2 cm away from the column bed.

A pH 9 buffer solution (0.05 *M*) is prepared from $Na_2CO_3$ and $NaHCO_3$ in deionized water. Also, a pH 5 buffer solution (0.05 *M*) is prepared from acetic acid and sodium acetate in deionized water. Amino acid stock solutions (1 μmol/ml) are prepared by dissolving a weighed amount of amino acid in the pH 9.0 buffer solution. Dabsyl chloride is prepared by dissolving a 33 mg amount in 10 ml of acetone. The solvent system is methanol–acetate buffer (60:40). The amino acids are purchased from Sigma, and other chemicals are purchased from Aldrich.

Dabsyl amino acids are prepared using a procedure reported previously [7]. One ml of amino acid stock solution and 1 ml of dabsyl chloride solution are mixed in a test tube, which is then stoppered and placed in a 80°C water bath until the reaction is completed (after 5 to 10 min). The reaction mixture is then evaporated by passing a nitrogen stream through it while heat is applied. The residue is then redissolved in 1 ml HPLC solvent mixture and stored in the freezer. An extraction procedure used to separate the derivatized dabsyl amino acids from unreacted dabsyl chloride [5,22] is also examined. After the derivatizing reaction between amino acids and dabsyl chloride is completed, acetone is evaporated from the reaction mixture with a gentle stream of nitrogen. The solution is then transferred to a separatory funnel and extracted twice with diethyl ether, and the organic layer is discarded. Solution pH

is adjusted to 3 using 0.5 *M* HCl, and the solution is then extracted five times with diethyl ether. At pH 3, dabsyl amino acids are extracted into the diethyl ether layer. The organic layers are combined and evaporated with nitrogen. The dabsyl amino acids are redissolved in 1 ml HPLC solvent mixture and stored in a freezer. Finally, the dabsyl amino acid solutions are used after filtering through a 0.22-μm filter.

## 3. Results and discussion

In an absorbing liquid solution, D4WM signal is generated more significantly from the thermally induced refractive-index grating than other nonlinear effects. The D4WM signal intensity $I_s$ can be described as [20]

$$I_s \propto f^2 Q^2 I_f I_b I_p (\alpha L/\cos\theta)^2 \quad (1)$$

In Eq. (1), $I_s$, $I_p$, $I_f$ and $I_b$ represent intensities of the phase conjugate signal, the probe beam, the forward pump beam and the backward pump beam, respectively. The parameter $f$ is inversely proportional to the analyte fluorescence quantum efficiency, $\alpha$ is the analyte absorption coefficient, $L$ is the analyte path length, $\theta$ is the angle between the forward pump and the probe beams.

The solvent parameter $Q$ is defined as: $Q = [2\pi(dn/dT)_p]/(\lambda\rho_0 C_p)$, where $(dn/dT)_p$ is the derivative of refractive index with respect to temperature at constant pressure, $\rho_0$ is the equilibrium value of the solvent density, $C_p$ is the specific heat at constant pressure and $\lambda$ is the excitation wavelength. Eq. (1) describes important characteristics of the D4WM signal including its cubic dependence on the laser intensity, and its quadratic dependence on the absorption coefficient, and hence, on the analyte concentration and molar absorptivity.

Fig. 2 shows the absorption spectra of dabsyl chloride and dabsyl proline in a pH 5 methanol–acetate buffer solution. Test chromatograms indicate that there is negligible amount of unreacted dabsyl chloride after dabsyl amino acid is extracted with diethyl ether. The absorbance of dabsyl proline at 488 nm (i.e., the argon ion laser line used for our detection) is about 86% of that at its maximum

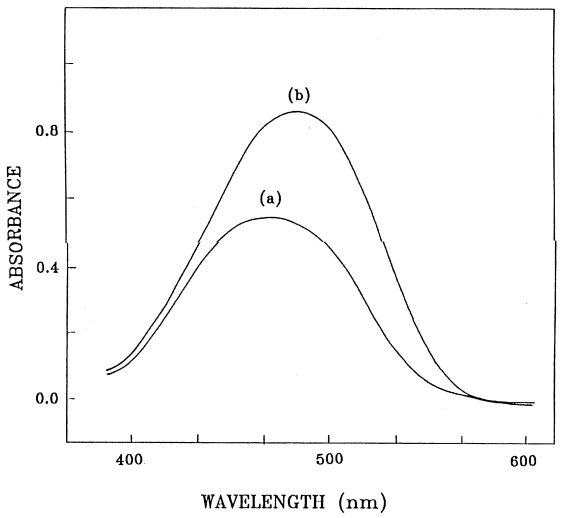


Fig. 2. Absorption spectra of (a) $3\cdot10^{-5}$ *M* dabsyl chloride in methanol–acetate buffer solution and (b) $5\cdot10^{-5}$ *M* dabsyl proline in methanol–acetate buffer solution.

absorption wavelength (465 nm). Hence, one could obtain better detection limits if an excitation wavelength that is closer to the analyte maximum absorption wavelength is used, since the D4WM signal has a quadratic dependence on analyte absorption coefficient. Nevertheless, the D4WM signal is still very strong even when excitation wavelength is off resonant as shown here and in our previous report for a cobalt analyte solution [21].

The effectiveness and the necessity of the dabsyl amino acid extraction step are also examined. For example, by extracting dabsyl amino acids, one could minimize background signal from unreacted derivatizing reagent, and hence, detect small amounts of components that are eluted early [5]. However, the chromatographic data of extracted dabsyl amino acids show that there is a negligible amount of unreacted dabsyl chloride after extraction with diethyl ether. In addition, optimum pH values for extraction of dabsyl amino acids with diethyl ether are from 2.6 to 5.0 [22]. In order to avoid unnecessary loss of dabsyl amino acids when a mixture of different dabsyl amino acids is extracted at a certain pH value, we choose not to use the extraction procedure.

Flow turbulence could affect the sharpness of the D4WM gratings, and hence, the signal strength [20].

When the flow-rate is extremely high, the analyte solution inside the laser probe volume may not have enough time to form the D4WM grating completely, and hence, the signal could be attenuated. On the other hand, at near-zero flow-rates or in static cells, sufficient time is available for the D4WM grating to form, and hence, maximum signal is obtained. However, analyte overheating and background optical noise levels due to thermal lens effect can be also high in static analyte solutions. When the analyte solution is flowed slightly, one can avoid overheating of the solution and minimize thermal-lens background signal, and hence, improve the D4WM signal-to-noise ratio. Experimental results show that grating formation times of most solution–solvent systems allow strong D4WM signals over a wide range of popular LC flow-rates. Flow-rates used in microbore LC systems are relatively slow, and one can expect to obtain complete formation of the D4WM grating, and hence, maximum signal strength. Optical background noise is then conveniently suppressed by the use of a lock-in amplifier. The effect of flow-rate on the conventional absorbance signal has been reported for protein separation using a 1-mm I.D. microbore column [23]. In our experiment, a flow-rate of 10 μl/min (i.e., the minimum flow-rate of our LC pump), and a 3-s lock-in amplifier time constant are used.

In a microbore HPLC system, the total volume of the column is very small, and hence, the extra-column volume caused by the tubing connections (between the column, the injection valve and the detector) could cause band dispersion, band broadening and peak attenuation. In order to minimize extra-column volume as much as possible in our D4WM detector interface, two small pieces of 1.5 cm×0.01 in. I.D. stainless steel tubing are used to connect the injection valve to the column, and the column to the capillary D4WM detector cell.

Fig. 3 shows a chromatogram of a mixture of four dabsyl amino acids detected by the nonlinear laser D4WM detector. Concentration of each of the dabsyl amino acids is $5\cdot10^{-5}$ *M*, and the injection volume is $2\cdot10^{-7}$ l (using partial injection technique), corresponding to 10 pmol of each dabsyl amino acid injected into the column. Since our emphasis is on the demonstration of D4WM as an effective HPLC detector, no attempt is made to optimize the chromatographic separation parameters. Using our D4WM detector, preliminary ''injected'' detection limits (at $S/N=2$) for glycine, alanine, methionine and proline are determined to be 0.78, 1.04, 1.29 and 1.10 pmol, respectively, using a relatively low total laser power for all input beams of 0.72 W.

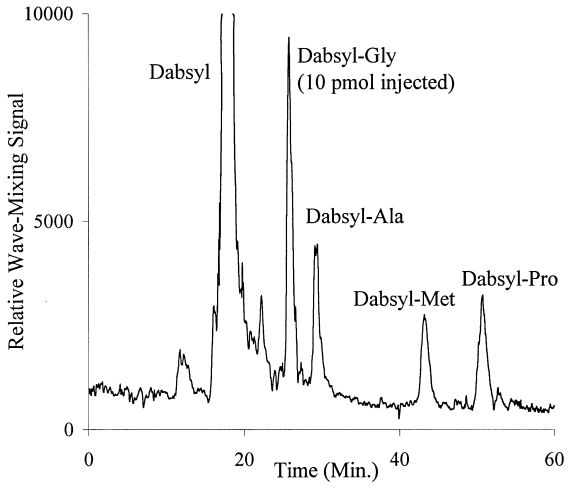


Fig. 3. Chromatogram of a mixture of four dabsyl amino acids detected by nonlinear laser degenerate four-wave mixing detector.

It is important to note that our detection sensitivity is affected by inadequate features of our current HPLC system, which is more suitable for large analytical columns than microbore columns. For instance, syringe pumps are superior to piston pumps for microbore liquid chromatography since a syringe pump can be used with flow-rates from 0.1 μl/min to 25 μl/min at a pressure of 10 000 p.s.i. (1 p.s.i.=6894.76 Pa). Furthermore, a narrower or shorter column could also improve the mass detection limit efficiently since the resulting void volume is smaller. A column that is smaller by a factor of three could improve the mass detection limit by a factor of ten [5]. Hence, the detection sensitivity of a D4WM detector in microbore HPLC could be further improved by using a shorter or narrower column.

Nonlinear laser D4WM offers many advantages as a HPLC or HPCE detector since it could detect both fluorescing and non-fluorescing analytes with excellent detection sensitivity. We have already demonstrated attomole-level detection sensitivity for strongly fluorescing analytes [20], and since the D4WM

signal is inversely proportional to the square of analyte fluorescence quantum yield, one could expect even stronger D4WM signal for non-fluorescing analytes. Unlike fluorescence methods, D4WM detection method allows virtually 100% optical signal collection efficiency since the signal is a laser beam that can be steered directly into a detector.

The D4WM detector measures absorbance values based on large absolute positive peaks measured against a virtually dark background, in contrast to conventional absorbance-based detection methods where a small difference of two large signals is measured. The D4WM optical alignment is relatively simple since the signal beam is visible to the naked eye, and once the alignment is set, it remains stable for an extended period of time. Furthermore, unlike other absorbance-based laser methods, only a single laser is needed to produce all the input beams in this one-color, one-laser D4WM detector system.

As in any optical methods, the background scattering noise is one of the limiting factors in optimizing the detection limit and successfully measuring the signal at very low concentration levels. These scattering noise levels could originate from the excitation light source itself, scattering of the source light off the optics, or any other optical noise that might be present near the detector. In our wave-mixing detection system, optical background noise sources include scattering of the pump and probe beams off the mirrors, beam splitters, lenses and other optics. These noise levels are adequately minimized using our amplitude-modulated detection system. The probe beam is intensity modulated effectively by an optical chopper, and the signal-to-noise ratio is significantly enhanced by the lock-in amplifier.

One of the major advantages of wave-mixing detection is the coherence property of the signal beam, and more importantly, its ability to propergate in a specific direction and space. This allows effective ''spatial filtering'', in addition to conventional optical or electronic filtering. For example, in a conventional optical absorption method, the signal is based on a small difference between two strong light intensities. However, in wave-mixing detection, the signal is a positive signal rising above a virtually dark background, i.e., the detector sees no light when the analyte is absent. Furthermore, since the laser-like signal beam has its own propagation direction that is different from those of the pump beams, optical interference from these beams is minimum at the detector. Hence, compared to conventional optical absorption methods, wave-mixing detection offers less background noise levels and better signal-to-noise ratios.

As shown in Eq. (1), the D4WM signal has a cubic dependence on the total laser intensity. This nonlinear dependency is one of the characteristic features that yield excellent detection sensitivity, since an order of magnitude increase in laser intensity results in three-orders of magnitude increase in signal. Background optical scattering noise can be effectively minimized by the use of small apertures in front of the detector, since the background noise is incoherent and the signal is a coherent laser-like beam. In addition, the availability of extremely strong signal intensity and virtually 100% signal collection efficiency further enhances the signal-to-noise ratio. Hence, the ''net increase'' in signal-to-noise ratio is better than many conventional laser-based methods where the analytical signal is not a laser beam. For instance, we have already demonstrated attomole-level detection sensitivity for liquid analytes as reported previously [20].

Since only a short absorption path length (e.g., less than 0.1 mm) is necessary in a D4WM detector to obtain excellent detection sensitivity, this technique is suitable for on-column detection of many popular chromatography or electrophoresis capillary columns. Using a lens with an appropriate focal length (e.g., less than 100 mm), one could focus the input beams to fit them all inside small capillary columns commonly used. Since the laser probe volume is small enough to interact only the fluid inside the column and not the glass walls, light scattering off the capillary tube is minimum. Hence, D4WM detection method is very effective as an ultrasensitive short-path-length on-column absorbance-based detector for many popular capillary chromatography or electrophoresis systems.

Furthermore, D4WM detectors require relatively low laser power levels (e.g., as low as a few milliwatts) for all input beams, and hence, small compact air-cooled continuous-wave lasers could be used to set up relatively compact HPLC or HPCE detectors. We have already demonstrated a detection limit of 2.5 ng/ml for lithium hyperfine structure

measurements using a pulsed laser energy as low as 10 nJ [18]. Hence, in addition to low-cost continuous-wave (CW) lasers, compact low-energy pulsed lasers could be used also to setup a simple on-column D4WM detector for many capillary chromatography or electrophoresis systems.

## 4. Conclusions

We have demonstrated D4WM as a sensitive absorbance detector for a microbore HPLC system. Advantages of D4WM detection over other laser-based detection methods include excellent optical signal collection efficiency, generation of signal in the form of a coherent laser beam, signal measurement against a virtually dark background, reliable detection of small absorbance values, excellent detection sensitivity comparable to laser-based fluorescence detectors, ability to detect both fluorescing and non-fluorescing analytes, relatively simple one-color one-laser optical setup, low power or energy requirements for continuous-wave or pulsed lasers and convenient on-column detection for many popular capillary columns of chromatography or electrophoresis systems. Works on other capillary-based D4WM detectors [24,25] suitable for chromatography and electrophoresis using even lower power lasers are under way.

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