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Note

Limitations of 2-diphenylacetyl-1,3-indandione-1-hydrazone as a pre-column fluorescence derivatization reagent

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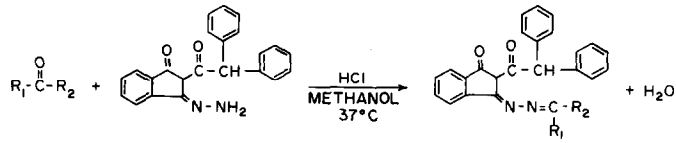
The qualitative and quantitative analysis of clinical and biomedical samples is a challenging problem that demands continual improvement of analytical instrumentation and methodology. The high complexity of such samples necessitates the improvement of separation methods, such as high-efficiency chromatographic and electrophoretic techniques. A promising technique which appears to be particularly well suited for the separation of complex biological samples is microcolumn liquid chromatography (LC)[1,2]. This technique is rapidly gaining recognition because of the very high separation efficiencies that can be achieved[3]. Other benefits of microcolumn LC include the reduced consumption of both sample and solvent[4], as well as the possibility of using novel detection methods[5–7].

Although many analytical techniques have been employed, laser-based detection methods seem especially promising for microcolumn LC[8]. The high intensity and narrow spectral bandwidth of the laser emission can provide sensitive and selective excitation using a wide variety of optical detection methods such as fluorescence, phosphorescence, polarimetry, refractometry, etc. Moreover, the highly collimated laser radiation can be readily focused into small-volume flowcells, as required for chromatographic detection, with minimal loss of radiant power. Although this combination of analytical techniques is still in the preliminary stages of development, microcolumn LC with laser-induced fluorescence detection appears to provide a powerful tool for the characterization of complex clinical and biomedical samples[9–12].

Not all molecules are naturally fluorescent at a given laser wavelength; hence, it may be necessary to chemically modify the analytes of interest to incorporate a fluorescent label[11–14]. In this study, we have examined the application of 2-diphenylacetyl-1,3-indandione-1-hydrazone (DPIH) as a fluorescent probe selective for ketone and aldehyde functional groups.



This reagent has been employed previously for qualitative identification in spot tests[15,16], and as a visualization agent in thin-layer chromatography[17,18]. In addition, Swarin and Lipari[19] have used DPIH for the identification of simple aliphatic ketones

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and aldehydes in automobile exhaust by LC. In this study, we have examined the use of DPIH as a derivatizing agent for ketosteroids, in conjunction with microcolumn LC separation and laser-induced fluorescence detection. This methodology will enhance the separation and selective detection of ketosteroids in the presence of other steroid classes, thus advancing clinical and biomedical analysis.

EXPERIMENTAL

Methodology

Standard solutions (10^{-5} *M*) are prepared by dissolving the ketosteroids (Sigma, St. Louis, MO, U.S.A.) in anhydrous methanol. A saturated solution (*ca.* $3 \cdot 10^{-4}$ *M*) of the derivatization agent is prepared by dissolving DPIH (Aldrich, Milwaukee, WI, U.S.A.) in anhydrous methanol. Equal volumes (3.0 ml) of the standard steroid and DPIH solutions are mixed together in a 10-ml vial, and 0.14 ml concentrated hydrochloric acid (12 *M*, J. T. Baker, Phillipsburg, NJ, U.S.A.) is added as catalyst. In some studies, aluminum(III) chloride, chromium(III) chloride, tin(II) and tin(IV) chlorides as well as magnesium bromide (Aldrich) are added as auxiliary catalysts. The reaction is allowed to proceed without stirring at 40°C for 30 min to 3 h. The solution is then clarified by using a syringe filtration system (Millipore, Bedford, MA, U.S.A.), and the solvent is evaporated under a stream of dry nitrogen. Although the derivatized steroids decompose in several hours at room temperature, they are stable for several days when stored without solvent at 0°C.

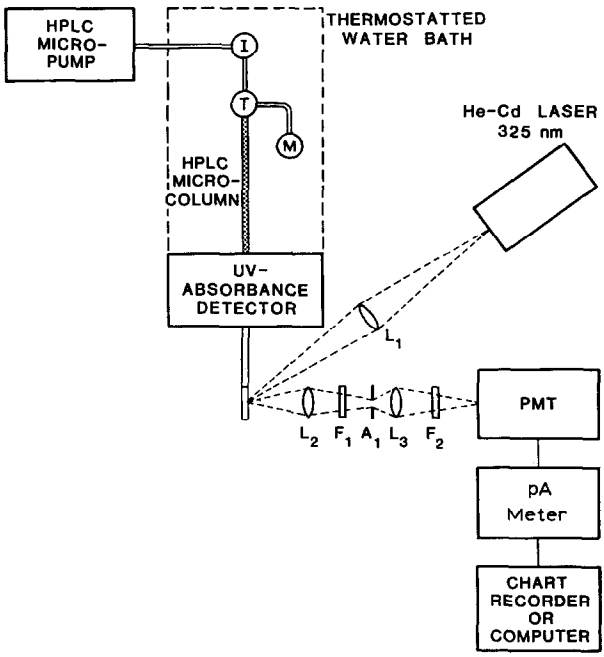


Fig. 1. Schematic diagram of the analytical system using microcolumn liquid chromatography with laser-induced fluorescence detection. I = Injection valve; T = splitting tee; M = metering valve for splitter; L = lens; F = filter; A = aperture; PMT = photomultiplier tube.

The derivatives are analyzed by high-efficiency microcolumn LC with detection by UV absorbance and laser-induced fluorescence methods. The analytical system is shown schematically in Fig. 1, and is described further in the following sections.

Chromatographic system

A single-piston reciprocating pump (Model 114M, Beckman, San Ramon, CA, U.S.A.) is used in the constant-pressure mode for solvent delivery. Samples are introduced by using a 1.0-μl valve injector (Model ECI4W1, Valco Instruments, Houston, TX, U.S.A.), after which the effluent is split (10:1 to 100:1) and applied to the chromatographic column. The microcolumn utilized for this study is prepared from fused-silica capillary tubing (89 cm × 200 μm I.D., Hewlett-Packard, Avondale, PA, U.S.A.) which is packed with an octadecylsilica material (Spheri-5 RP-18, 5 μm, Brownlee Labs., Santa Clara, CA, U.S.A.), as described previously[20]. These microcolumns approach the theoretical limits of efficiency, and are capable of achieving theoretical plate numbers in excess of 100 000 under standard test conditions[20].

UV absorbance detector

A variable-wavelength UV-absorbance detector (Model Uvidec 100-V, Jasco, Tokyo, Japan) is modified for microcolumn LC by using a 25-nl fused-silica flowcell (0.5 cm × 80 μm I.D.). Light throughput from the deuterium lamp source is limited by razor-blade slits mounted parallel to the capillary flowcell.

Laser fluorescence detector

A helium–cadmium laser (Model 3112-10S, Omnichrome, Chino, CA, U.S.A.), with approximately 10 mW of continuous-wave output at 325 nm, is used as the excitation source. The laser radiation is focused directly upon the optically transparent fused-silica microcolumn using a fused-silica lens (2.8 cm diameter, 10.0 cm focal length, A612810, Esco Products, Oak Ridge, NJ, U.S.A.). Sample fluorescence is

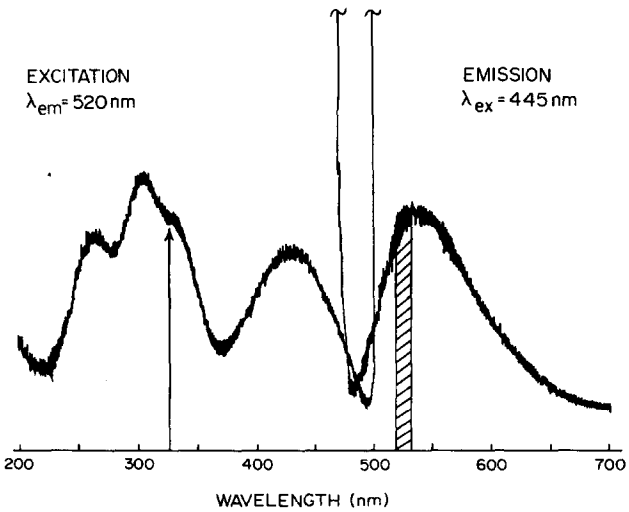


Fig. 2. Fluorescence excitation and emission spectra of ketosteroids derivatized with DPIH in methanol.

collected perpendicular and coplanar to the excitation beam with a fused-silica lens (2.54 cm diameter, 2.54 cm focal length, A110010, Esco Products). The emission is then isolated by appropriate interference filters (FS-204-F, Corion, Holliston, MA, U.S.A.) and is focused onto a photomultiplier tube (Centronic Model Q4249B, Bailey Instruments, Saddle Brook, NJ, U.S.A.). The resulting photocurrent is amplified by a picoammeter (Model 480, Keithley Instruments, Cleveland, OH, U.S.A.) and displayed on a chart recorder (Model 585, Linear Instruments, Reno, NV, U.S.A.).

In preliminary studies, the excitation and emission spectra of the steroid derivatives in methanol were monitored by using a conventional fluorimeter (Model 512, Perkin-Elmer, Norwalk, CT, U.S.A.) with a Xe lamp source. Typical fluorescence spectra are shown in Fig. 2, together with the excitation (325 nm) and emission (520 nm) wavelengths selected for chromatographic analysis. The spectral properties of the steroid derivatives correlate well with those reported previously for the acetone derivative[17], which has maximum absorbance at 297 and 412 nm (ε_{max} 38 800 and 7060 l/mol cm, respectively) and maximum fluorescence emission at 570 nm (φ_F 0.1–0.3).

RESULTS AND DISCUSSION

In initial experiments, fluorescence spectroscopy was used to confirm the formation of fluorescent products upon reaction of the DPIH reagent with simple aliphatic and aromatic ketones. Subsequently, a series of mono-, di-, and tri-ketosteroids was examined under the same reaction conditions. In general, the derivatization agent was reactive with all ketosteroids examined and formed highly fluorescent products. Steroids with conjugated and aromatic ketone sites were found to be far more reactive than the corresponding unconjugated sites. Furthermore,

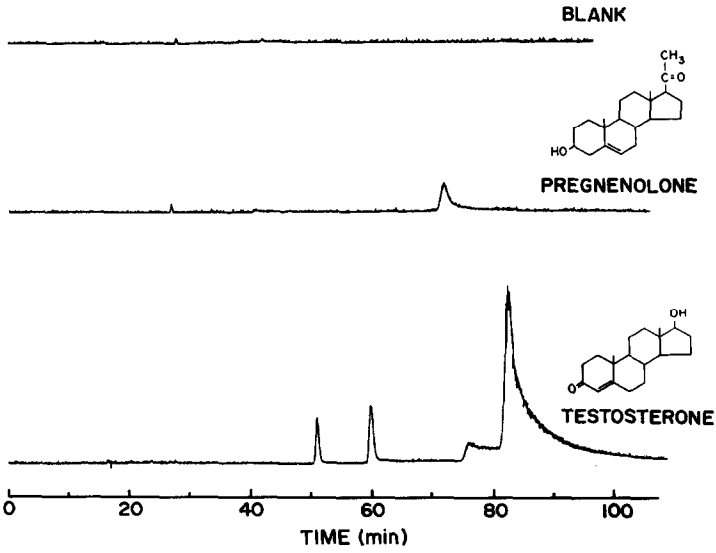


Fig. 3. Chromatograms of pregnenolone and testosterone derivatized with DPIH. Microcolumn: 89 cm × 200 μm I.D. fused-silica capillary, packed with Spheri-5 RP-18. Mobile phase: methanol at 0.9 μl/min. Fluorescence detector: 325 nm excitation, 520 nm emission, sensitivity 1.0 μA/V, 0.1 V full scale.

sterically hindered ketone sites within the steroid rings were less reactive than accessible sites on the side chain. Finally, no fluorescent products were formed for steroids which contained hydroxyl groups but no ketone functionalities.

These preliminary results seemed quite promising, as they appeared to indicate that selective analysis of ketosteroids was feasible using the DPIH reagent. Upon chromatographic separation, however, multiple fluorescent products were observed for monoketosteroids (Fig. 3) as well as polyketosteroids (Fig. 4). Although this phenomenon has been widely reported in the literature for other reagents (refs. 21,22, and references cited therein), it is generally ascribed to the formation of two *syn* and *anti* isomers. With the present reagent, four products were formed with greatly differing chromatographic and spectroscopic properties, as exemplified by testosterone (Fig. 3). Two products, of approximately equal and low concentration, were slightly retained with good peak shape under these chromatographic conditions and showed maximum absorbance at 300 nm wavelength. The other two products, generally of higher and unequal concentration, were more strongly retained with strong tailing under these chromatographic conditions and showed maximum absorbance at 320 nm. Because of the differing chromatographic and spectroscopic

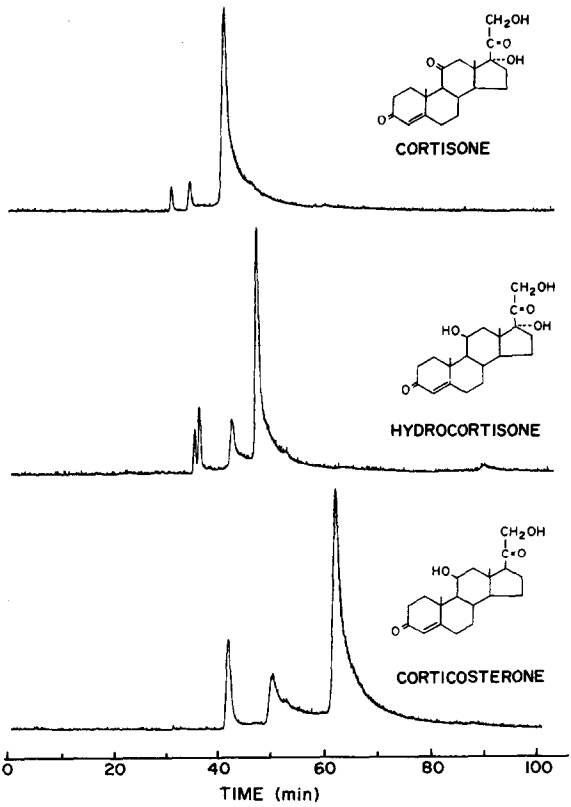


Fig. 4. Chromatograms of cortisone, hydrocortisone, and corticosterone derivatized with DPIH. Chromatographic conditions are described in Fig. 3.

properties, it was initially believed that the multiple products were the results of impurities in the reagents or by-products of the derivatization reaction. Hence, an extensive purification of the materials was performed, followed by a thorough optimization of the reaction conditions using testosterone as a model solute.

Catalyst studies

Several organic and inorganic acids of varying strength were compared as catalysts for the reaction of testosterone with DPIH. No fluorescent products were observed without a catalyst or with a weak organic acid catalyst such as acetic acid. Mild inorganic acids, such as boron trifluoride, appeared to catalyze the reaction but yielded multiple fluorescent products. Similar results were observed for strong mineral acids, such as hydrochloric and sulfuric acids. Although the initial rate of reaction was faster with sulfuric acid than with hydrochloric acid, the decomposition rate of the derivatives was also increased. Hence, hydrochloric acid was chosen for further catalytic studies of the reaction conditions.

Because the rate of addition of the hydrazine nucleophile to the carbonyl group is pH dependent[22,23], the effect of catalyst concentration was examined. The concentration of hydrochloric acid was varied between 0.02 and 0.20 *M*, as shown in Fig. 5, and the optimal concentration appeared to be approximately 0.08 *M*. The amount of catalyst influenced the rate of product formation and decomposition, but did not influence the number of reaction products.

Various Lewis acids, such as $AlCl_3$, $CrCl_3$, $SnCl_2$, $SnCl_4$, and $MgBr_2$ were added as auxiliary catalysts with hydrochloric acid. The Lewis acids were expected to complex selectively with the conjugated diketone group of the DPIH reagent, possibly reducing the number of by-products formed in the derivatization reaction[24]. Although significant differences were observed in the rates and relative amounts of products formed (*vide infra*), Lewis acids were not successful in eliminating the multiple fluorescent products.

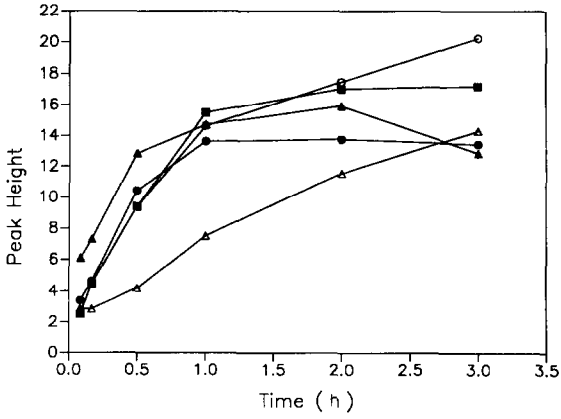


Fig. 5. Effect of hydrochloric acid catalyst concentration on derivatization of testosterone with DPIH. Testosterone $5.2 \cdot 10^{-5}$ *M*; DPIH $3.0 \cdot 10^{-4}$ *M*; catalysts: (△) 0.02 *M* HCl, (○) 0.04 *M* HCl, (■) 0.08 *M* HCl, (●) 0.16 *M* HCl, (▲) 0.20 *M* HCl; temperature 40°C.

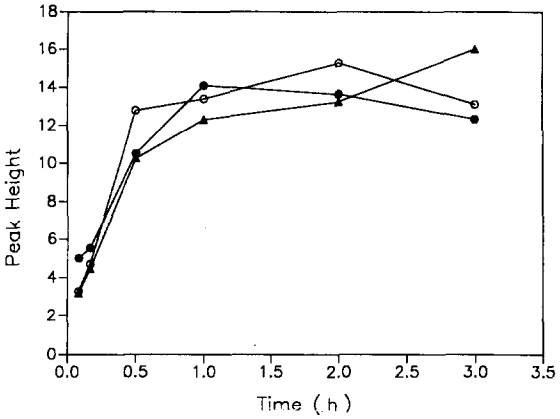


Fig. 6. Effect of temperature on derivatization of testosterone with DPIH. Testosterone $5.2 \cdot 10^{-3}$ *M*; DPIH $3.0 \cdot 10^{-4}$ *M*; catalyst 0.08 *M* HCl; temperatures: (●) 30°C; (▲) 40°C; (○) 50°C.

Finally, to determine whether specific interaction of the catalyst with either the derivatizing agent or the steroid was responsible for the multiplicity of reaction products, the order and timing of reagent addition were systematically varied. However, no change in the amount or number of reaction products was observed.

Temperature studies

The derivatization reaction of testosterone was examined as a function of temperature in the range of 30 to 50°C. As illustrated in Fig. 6, no significant difference was observed in the rate of product formation or decomposition within this temperature range. This indicates that the rate-limiting step in the derivatization reaction is equilibrium controlled, as expected[23], rather than kinetically controlled. Furthermore, the number of reaction products was invariant with temperature, indicating that thermal decomposition of the steroid or steroid derivative was not the source of multiple products.

Additional studies

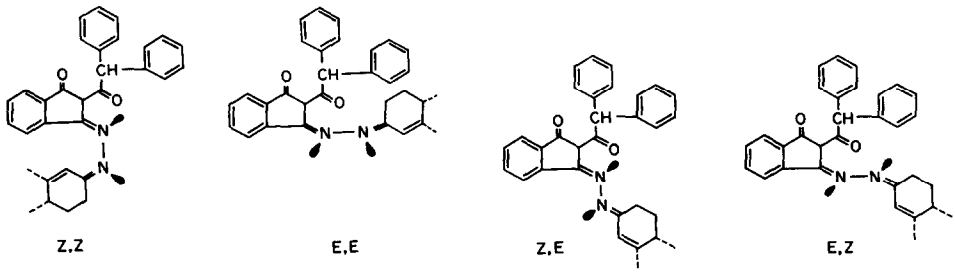
Since water is a product of the derivatization reaction (see Introduction), it may act as a stimulus for product decomposition. This possibility was examined by dehydrating all reagents, solvents, and solutions with a drying agent such as calcium chloride, sodium sulfate, or molecular sieve 4A. No change was observed in the amount or multiplicity of products formed in the presence or absence of water.

Another possible source of interference is the formation and subsequent derivatization of additional ketone sites by tautomerization of hydroxyl groups. This possibility was examined by reacting cholesterol, a steroid with a hydroxyl group in the C-3 position, with the DPIH reagent. No fluorescent products were formed; hence, the possibility of keto–enol tautomerization under the present reaction conditions seems remote.

Finally, an attempt was made to isolate and identify the multiple products formed in the derivatization of testosterone. Fractions corresponding to each of the four products were collected from the liquid chromatograph and reanalyzed to

confirm purity. Mass spectrometric analysis revealed that each fraction had the same molecular ion (m/z 624) and similar fragmentation patterns. Upon examination of the fractions by LC, it was discovered that each fraction contained all four products formed in the original reaction mixture. Hence, a dynamic relationship must exist between the reaction products such that interconversion, whether by rearrangement or by isomerization, is possible in the presence of a suitable catalyst.

The most probable source of such isomeric products is the formation of configurational isomers at the azine bond. For simple aliphatic ketones and steroids such as pregnenolone (Fig. 3) that react on the flexible side chain, the configurational isomers may or may not be chromatographically resolved. For steroids such as testosterone (Fig. 3) and the corticosteroids (Fig. 4), however, the rigid ring structure at the C-3 ketone site causes great structural dissimilarity. Four possible *E* and *Z* configurations, shown below, may arise upon derivatization of such steroids with DPIH.



It is possible that the two early eluting derivatization products of testosterone (see Fig. 3) are the *E,E* and *E,Z* configurations. From the examination of molecular models, these products appear to be sterically hindered, and are not expected to be formed in abundance. Furthermore, the lone pairs of electrons on the azine and carbonyl groups are not completely accessible, so tailing of chromatographic peaks due to strong adsorption on silanol groups is not expected and is not observed. Presumably, the later eluting products of testosterone are the *Z,Z* and *Z,E* configurations. From examination of molecular models, the *Z,E* configuration is most stable and is probably the most retained peak, while the other predominant peak is the *Z,Z* conformer. The lone pairs of electrons on the azine and carbonyl groups in these configurational isomers are readily accessible, so concerted adsorption (tailing) would be expected and is observed. To substantiate these configurational assignments, a weak Lewis acid ($MgBr_2$) was added during the derivatization. The *Z,Z* and *Z,E* configurations with accessible electron pairs are expected to bind weakly with the Lewis acid, which will stabilize and enhance formation of these products. Conversely, the *E,E* and *E,Z* configurations which lack the accessible electron pairs cannot be stabilized in this manner. As predicted, a substantial increase in product amount was observed for the later eluting products, whereas no change was discernible for the early eluting products. Hence, the proposed assignment of configurational isomers appears to be justified.

CONCLUSIONS

The reagent DPIH is very reactive and forms highly fluorescent derivatives with simple ketones as well as mono-, di-, and triketosteroids. Unfortunately, its utility as a precolumn derivatizing agent appears to be limited by the formation of multiple configurational isomers. This reagent remains useful as a spray reagent in thin-layer chromatography and is potentially useful as a post-column derivatization agent, where the presence of multiple products is inconsequential.

ACKNOWLEDGEMENTS

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REFERENCES

1 M. Novotny, *Anal. Chem.*, 53 (1981) 1294A.
2 M. Novotny, *LC · GC Mag. Liq. Gas Chromatogr.*, 3 (1985) 876.
3 H. G. Menet, P. C. Gareil and R. H. Rosset, *Anal. Chem.*, 56 (1984) 1770.
4 V. L. McGuffin, *LC · GC Mag. Liq. Gas Chromatogr.*, 2 (1984) 282.
5 V. L. McGuffin and M. Novotny, *Anal. Chem.*, 53 (1981) 946.
6 V. L. McGuffin and M. Novotny, *Anal. Chem.*, 55 (1983) 2296.
7 K. Jinno, S. Nakanishi and C. Fujimoto, *Anal. Chem.*, 57 (1985) 2229.
8 E. S. Yeung, *Adv. Chromatogr.*, 23 (1984) 1.
9 E. J. Guthrie, J. W. Jorgenson and P. R. Dluzneski, *J. Chromatogr. Sci.*, 22 (1984) 171.
10 J. Gluckman, D. Shelly and M. Novotný, *J. Chromatogr.*, 317 (1984) 443.
11 V. L. McGuffin and R. N. Zare, *Appl. Spectrosc.*, 39 (1985) 847.
12 S. Folestad, B. Galle and B. Josefsson, *J. Chromatogr. Sci.*, 23 (1985) 273.
13 V. L. McGuffin and R. N. Zare, *ACS Symp. Ser.*, 297 (1986) 120.
14 V. L. McGuffin and R. N. Zare, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 8315.
15 R. Brandt, J. C. Kouines and N. D. Cheronis, *Microchem. J.*, 6 (1962) 519.
16 R. A. Braun and W. A. Mosher, *J. Am. Chem. Soc.*, 80 (1958) 3048.
17 D. J. Pietrzyk and E. P. Chan, *Anal. Chem.*, 42 (1970) 37.
18 E. J. Poziomek, E. V. Crabtree and J. W. Mullin, *Anal. Lett.*, 14(A11) (1981) 825.
19 S. J. Swarin and F. Lipari, *J. Liq. Chromatogr.*, 6 (1983) 425.
20 J. C. Gluckman, A. Hirose, V. L. McGuffin and M. Novotny, *Chromatographia*, 17 (1983) 303.
21 J. Drozd, *Chemical Derivatization in Gas Chromatography (Journal of Chromatography Library*, Vol. 19), Elsevier, Amsterdam, 1981, pp. 92–97, 151–165.
22 L. A. Sternson, in R. W. Frei and J. F. Lawrence (Editors), *Chemical Derivatization in Analytical Chemistry*, Vol. 1, Plenum Press, New York, 1981, pp. 183–193.
23 T. H. Lowry and K. S. Richardson, *Mechanism and Theory in Organic Chemistry*, Harper and Row, New York, 1976, pp. 432–439.
24 A. L. Gemal and J. L. Luche, *Tetrahedron Lett.*, 22 (1981) 4077.