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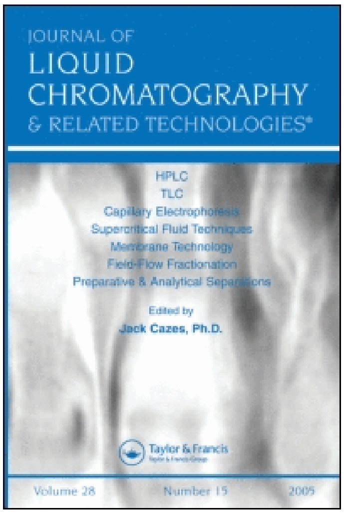

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2, 5-Dihydroxybenzohydrazide as Electroactive Labeling Reagent for Aliphatic Aldehydes by High Performance Liquid Chromatography with Electrochemical and Ultraviolet Detection

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(10), 1933-1945 (1995)

2,5-DIHYDROXYBENZOHYDRAZIDE AS ELECTROACTIVE LABELING REAGENT FOR ALIPHATIC ALDEHYDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL AND ULTRAVIOLET DETECTION

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ABSTRACT

A method was developed for the analysis of aldehydes by high performance liquid chromatography coupled with electrochemical and ultraviolet detection. The electrochemical oxidation of carbonyl compounds derivatized with 2,5-dihydroxybenzohydrazide (2,5-DHBH) was investigated at porous graphite electrodes. The compounds were separated on an Adsorbosphere column with a methanol-acetonitrile-phosphate buffer eluent and detected at graphite electrodes set at an oxidation potential of +0.3 V. The influence of the mobile phase buffer concentration and pH on the detector response was also studied. The electrochemical detection was compared with conventional UV detection and was found to achieve enhanced specificity and sensitivity (S/N=3). The

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derivatization was shown to be quantitative and the response linear between 1-15 ng/ml. The method is rapid, reproducible and the detection limit is 130 fmol for an injection volume of 5 µl with cyclohexanecarboxaldehyde as internal standard (I.S.). This technique is applicable to the assay of carbonyl compounds in flavor chemistry.

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INTRODUCTION

The determination of carbonyl compounds in analytical chemistry is becoming always more important for the monitoring of formation reactions or the transformation of aldehydes [1] and for the study of the fractions of the volatile compounds responsible for the organolectic characteristics of natural aromas and flavoring agents [2,3].

The analysis of microquantities of compounds implicated in these mechanisms allows the study of the trend of oxidative degradation phenomena, change in flavor, racemization, and to carry out controls on the genuineness in the field of essential oils and aromas [4].

The normal techniques for the determination of carbonyl compounds are GC [5], Headspace CGC [6], and GC coupled with MS and FTIR [7]. Furthermore, the technique of derivatization for spectrophotometric [8] and spectroflurometric HPLC analysis was used [9,10]. We also proposed a sensitive and specific method for the evaluation of aldehydes, octanal and decanal, which are responsible for the organolectic characteristics of essential citric oils and flavoring agents. To this end we established a new reaction of derivatization of these two aldehydes and cyclohexane-carboxaldehyde, as internal standard (I.S.), with 2,5-dihydroxybenzo-hydrazide (2,5-DHBH) to form electroactive hydrazone measurable by

HPLC with electrochemical detection (ED). This method, moreover, could be applied to the analysis of cosmetic products [11], topical formulations [12] and medical bactericides [13] containing octanal and decanal.

EXPERIMENTAL

Apparatus

The HPLC apparatus comprised two Model 510 pumps, a Model 712 WISP auto-injector and a Model 490E absorbance detector (Waters Assoc., Milford, MA, USA) set at 257 nm and 0.05 absorbance units full scale. The UV detector was connected in series with the electrochemical detector (Model 5100A Coulochem; ESA, Bedford, MA, USA) which consisted of a control module and an analytical cell (Model 5010) containing two in-line porous graphite coulometric electrodes.

The analysis was performed in the oxidative mode. The ED sensitivity range and response time were set at 100 nA and 10 s, respectively. Signals from the detectors were converted to chromatographic traces and integrated by an APC IV computer system (NEC, Boxborough, MA, USA) using Maxima 820 software (Waters Assoc., Milford, MA, USA).

All melting points are uncorrected. Mass spectra were obtained on a model Kratos MS 25 RF. IR spectra were recorded on a Perkin-Elmer 1600 Fourier transformed spectrometer as KBr disks. Elemental analysis for C, H, N were obtained on a Carlo Erba 1106 analyzer (Milan, Italy). UV absorption spectra were recorded on a Uvikon 860 (Kontron, Zurich, Switzerland) spectrometer in CH_3CN/MeOH 9:1 solution. Analytical thin

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layer chromatography (TLC) was performed on Merck 60 F-$_{254}$ silica gel plates.

Chemicals

2,5-dihydroxybenzoic acid, cyclohexanecarboxaldehyde, octanal, decanal and KH_2PO_4 were obtained from Fluka (Buchs, Switzerland). HPLC-grade methanol and acetonitrile were from Carlo Erba (Milan, Italy). Milli-Q water (Millipore, Bedford, MA, USA) was used. Other chemicals used were of reagent grade or better.

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Chromatographic conditions

Separations were performed on a 3 μm Adsorbosphere column (100x4.6 mm i.d.; Alltech, Deerfield, IL, USA) fitted with a guard column (Hypersyl ODS RP-18, 5 μm particles, 4x4 mm i.d.; Policonsult, Rome, Italy) and eluted, isocratically, with acetonitrile-0.05 M phosphate buffer-methanol (39/39/22, v/v/v) adjusted to pH 7.0 with KOH. The mobile phase was filtered through GS-type filters (0.22 mm, Millipore, Bedford, MA, USA) and on-line degassed with a Model ERC-3311 solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at ambient temperature, at a flow-rate of 1.0 ml/min. Peak areas were quantified by the internal standard.

Synthesis

2,5-dihydroxybenzohydrazide (2,5-DHBH,**1**) was prepared in a similar manner to that described in literature [14,15].

UV: λ_{max} 327 nm, $\varepsilon = 5{,}233\ M^{-1} \cdot cm^{-1}$.

Cyclohexanecarboxaldehyde hydrazone of 2,5-DHBH (CY-2,5-DHBH, **3a**) 2,5-DHBH (80 mg, 0.48 mmol) and cyclohexanecarboxaldehyde **2a** (90 μl, 0.48 mmol) were dissolved in methanol (5 ml) and acetic acid (1 ml). The mixture was stirred for 1 h at room temperature and then water was added. A precipitate was collected, washed and dried. The solid product was crystallized from methanol-water gave CY-2,5-DHBH as colorless needles (92 mg, 73%) m.p. 137-139°C.

Anal. Calc'd. for $C_{14}H_{18}N_2O_3$ (M.W. 262.31): C, 64.11; H, 6.92; N, 10.68. *Found*: C, 63.97; H, 6.95; N, 10.65. MS (IE 70 eV) *m/e*: 262 (M^+), 152 (M^+-$C_6H_{11}CH{=}N$), 137 (M^+- $C_6H_{11}CH{=}N{-}NH$). IR (KBr) ν_{max} (cm^{-1}): 3450, 3234, 2913, 1625, 1548, 1449. UV: λ_{max} 257 nm, ε_1 = 11,150 $M^{-1}\cdot cm^{-1}$; λ 337 nm, ε_2 = 5,375 $M^{-1}\cdot cm^{-1}$.

Octanal **and** ***Decanal hydrazone of 2,5-DHBH*** (OC-2,5-DHBH and DE-2,5-DHBH respectively) were prepared by the same procedure described above for CY-2,5-DHBH. The elemental analysis and the melting point data for new compounds are described below.

OC-2,5-DHBH (**3b**): White needles (70 mg, 63%) m.p. 144 -145°C.

Anal. Calc'd for $C_{15}H_{22}N_2O_3$ (M.W. 278.35): C, 64.76; H, 7.90; N, 10.06. *Found* : C, 64.58; H, 7.45; N,10.23. MS (IE 70 eV) *m/e*: 278 (M^+), 194 (M^+ -$(CH_2)_5CH_3$), 137 (M^+- $CH_3(CH_2)_6CH{=}N{-}NH$). IR (KBr) ν_{max} (cm^{-1}): 3381, 2947, 1637, 1577. UV: λ_{max} 257 nm, ε_1 = 11,924 $M^{-1}\cdot cm^{-1}$; λ 334 nm, ε_2 = 5,691 $M^{-1}\cdot cm^{-1}$.

DE-2,5-DHBH (**3c**): Light pink needles (90 mg, 69%) m.p. 151 -152°C.

Anal. Calc'd for $C_{17}H_{26}N_2O_3$ (M.W. 306.41): C, 66.64; H, 8.55; N, 9.14. *Found:* C, 66.52; H, 8.50; N, 9.53. MS (IE 70 eV) *m/e*: 306 (M^+), 194 (M^+ -$(CH_2)_7CH_3$), 137 (M^+- $CH_3(CH_2)_6CH{=}N{-}NH$). IR (KBr) ν_{max} (cm^{-1}): 3197,

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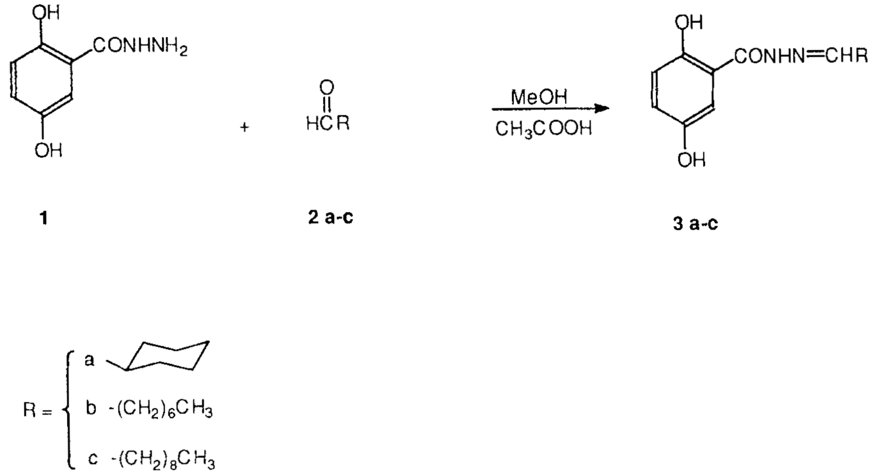

Scheme 1: Reaction of aldehydes **2 a-c** with 2,5-DHBH to give hydrazone derivatives **3 a-c**

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2924, 1643, 1588. UV: λ_{max} 257 nm, $\varepsilon_1 = 12{,}258\ M^{-1} \cdot cm^{-1}$; λ 335 nm, $\varepsilon_2 = 5{,}411\ M^{-1} \cdot cm^{-1}$.

RESULTS

Scheme 1 illustrates the reaction of aldehydes **2 a-c** with 2,5-DHBH to give hydrazone derivatives **3 a-c.** Experiments were performed to determine optimum derivatization time in order to give maximum conversion of **2 a-c** to their electroactive derivatives. Figure 1 shows the trend of the derivatization procedure which was complete after 60 min with 90% yield and the compounds show the stability in the reaction mixture until 24 h after the optimum. This derivatization procedure

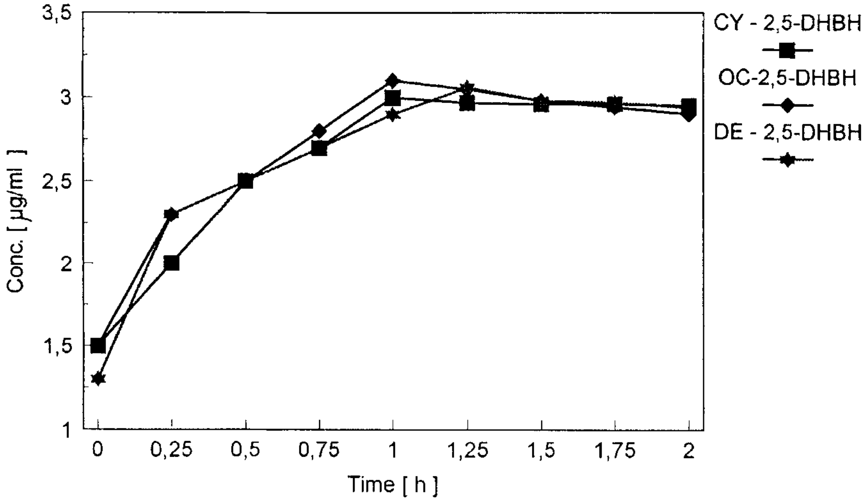

Figure 1: Concentration of CY-2,5-DHBH **3 a-c** *versus* time curve

achieves enhanced sensitivity. The linearity of the method was also determined: five standards containing concentrations of **3 a-c** in the range 1-15 ng/ml. The calibration curve of I.S. showed a correlation coefficient of 0.9997 and the equation of the linear regression was $y= 0.0679 + 0.5076x$ where x was the area ($\cdot 10^6$) under the peak. Typical chromatograms of UV and ED detection of standards are shown in fig. 2 and 3 respectively, the analysis was complete within 10 minutes and the retention times were 1.65, 2.54, 4.23 and 9.01 for 2,5-DHBH, CY-2,5-DHBH, OC-2,5-DHBH and DE-2,5-DHBH; the injected volume and concentrations were 5 µl and 100 pmol/ml, respectively. Detection limit was determined from five runs and was in the range of 100-200 fmol per injection (S/N= 3). These values are significantly lower (at least five times) than those registered for conventional UV detector (fig. 2). The

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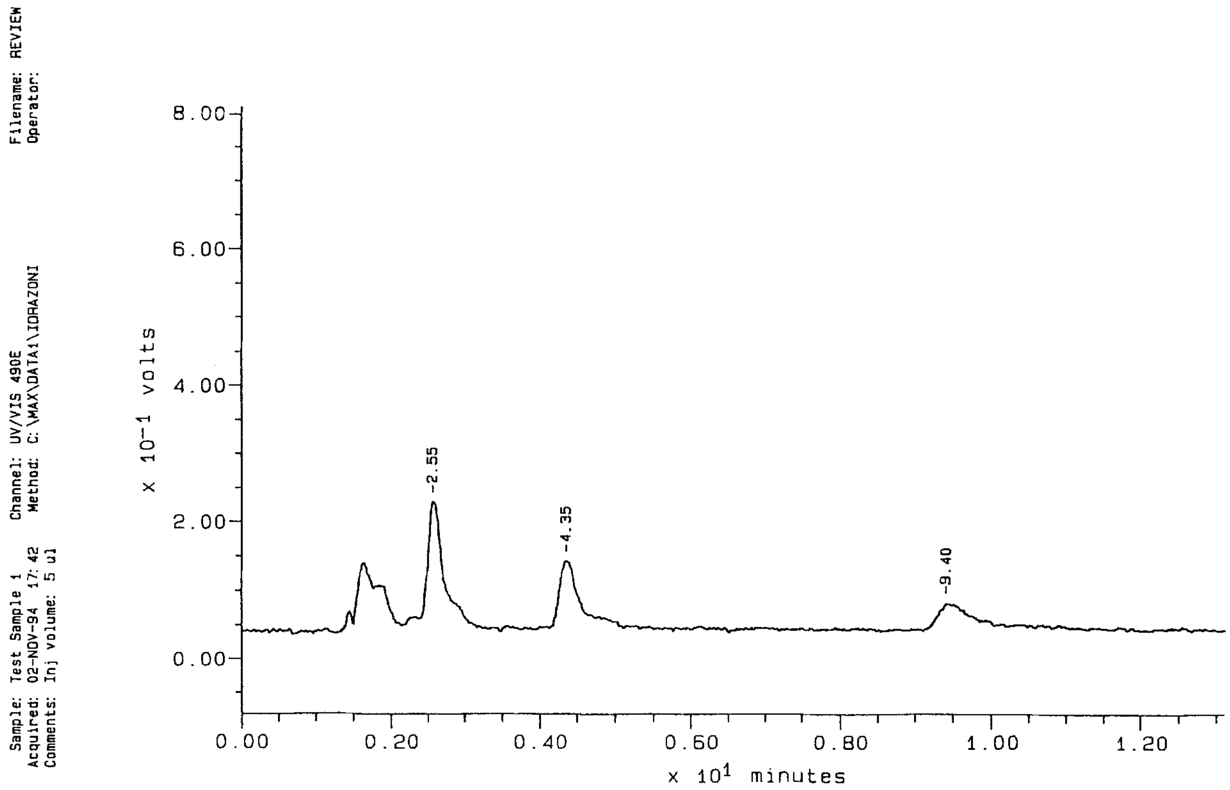

Figure 2: Chromatogram obtained with UV detector fixed at 257 nm by injection of 5 µl of the standard solution of **1** and **3 a-c** (100 pmol/ml)

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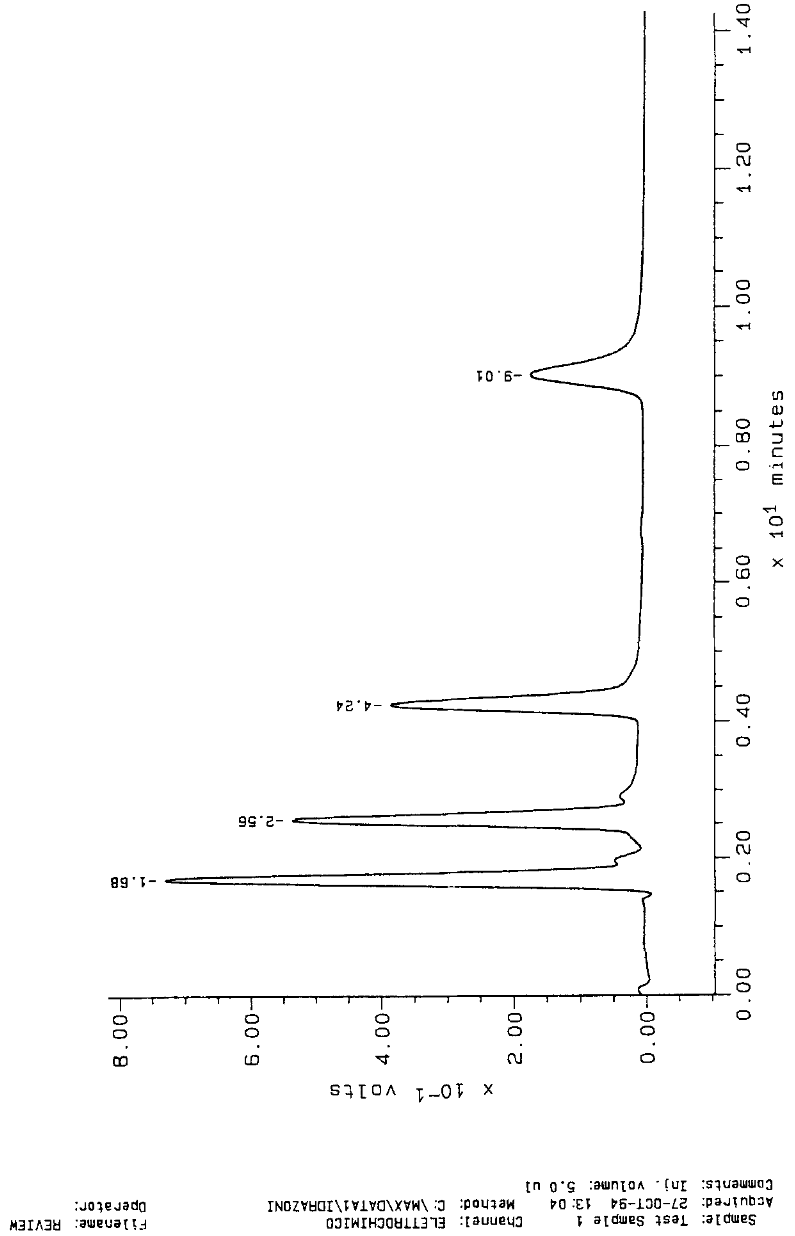

Figure 3: Chromatogram obtained with ED detection obtained by injection of 5 µl of the standard solution of **1** and **3 a-c** (100 pmol/ml)

derivatization was shown to be over 90% and the response linear between 1-15 ng/ml.

DISCUSSION

HPLC coupled with electrochemical detection was used to determine trace amounts of electroactive compounds because of its high sensitivity and selectivity [16]. Moreover, enhanced selectivity is achieved by HPLC-ED in this method without blank interference due to the limited number of substances which can undergo redox reactions under this condition. Several parameters were examined in order to optimize the electrochemical detection of hydrazone derivatives. Preliminary experiments indicated that under the chromatographic conditions reported above, the **3 a-c** derivatives responded at the ED at oxidation potentials lower than +0.8 V. With additional applied potential, no further increase in hydrazone peak heights occurred and a rise in the background current was observed. Electroactive properties of the derivatives **3 a-c** were examined with their hydrodynamic voltammograms (fig. 4), the figure indicates that the best potential is +0.3 V, because a superior potentials there would be an amplified response only for the derivatization reagent (2,5-DHBH), which could mask in the chromatogram the result of the I.S. that has a retention time of 2.54 min.

The ED performance was markedly influenced by the ionic strength but not by the pH of the mobile phase. With increasing concentrations of the phosphate buffer (from 0.01 to 0.05 M), an increase of the hydrazone

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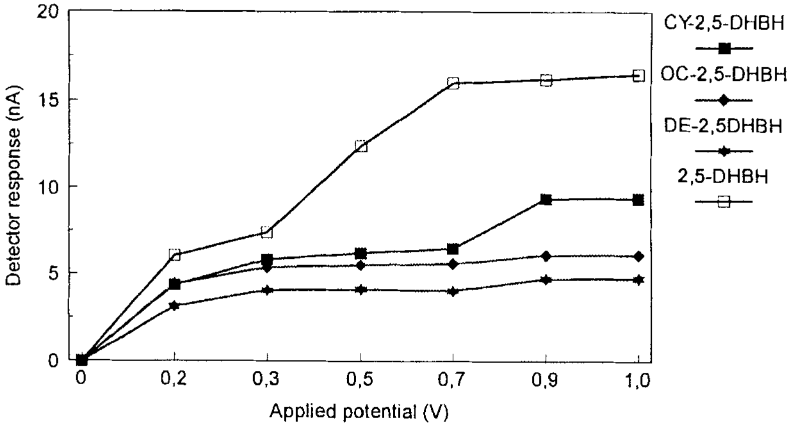
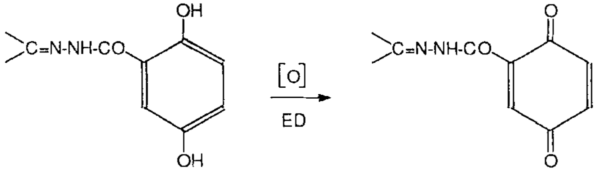


Figure 4: Hydrodynamic voltammograms of the electroactive derivatives **3 a-c**



Scheme 2: General oxydative mechanism of electroactive Schiff bases **3 a-c** at porous graphite electrodes.

electrochemical response was observed. No significant improvement in the detector response was achieved by further increasing the phosphate molarity, which was consequently fixed at 0.05 M and the pH at 7.0.

This method offers the possibility of determining octanal and decanal with a simple reaction of derivatization and formation of electroactive Schiff bases using cyclohexanecarboxaldehyde as internal standard. The chromatographic analysis with UV detection fixed at 257 nm (Fig. 2) of the

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standard solution indicates that the method is also applicable to conventional UV detectors but surely for concentrations >100 pmol/ml. The applied potential, as reported in literature [17], selectively oxidizes the hydroquinone derivatives (**3 a-c**) according to the following reaction (Scheme 2). In conclusion, 2,5-dihydroxybenzohydrazide can easily be used as electroactive labeling reagent for aliphatic aldehydes by high performance liquid chromatography with electrochemical detection.

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