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New type of microfabricated carbon electrodes for high-performance liquid chromatography—Amperometric detection of fat-soluble vitamins and antioxidants

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ABSTRACT

This study evaluates performance of thin-film carbon electrodes prepared by physical vapor deposition of the electrode material on a polyetheretherketone substrate and compares their performance with that of a standard type of glassy carbon electrodes for chromatographic detection cell of thin-layer type. Kaempferol, retinol, retinyl acetate, cholecalciferol, α-tocopherol and γ-tocopherol were selected for the study and their respective detection limits were found to be 0.63, 5.84, 5.91, 28.19, 16.80 and 16.11 pg. The calibration plots were linear for at least three orders of magnitude for all of the six analytes selected for evaluation. Hydrodynamic voltammograms are shown for both types of electrodes for the range of working electrode potentials between +0.40 and +1.40 V. Also shown and discussed are plots of signal-to-noise vs. detection potential. The working electrode potential of +1.30 V was found to be required for an improved long-term stability of detection performance and was suitable for the detection of all six analytes.

1. Introduction

Fat-soluble vitamins have been analyzed for many purposes in a variety of sample matrices [1,2]. Among the most important assays are naturally occurring vitamins in food and beverages, vitamins in dietary supplements and vitamins in biological samples. Biological microsamples such as tissue from autopsies are analyzed in order to clarify the role of vitamins and other antioxidants in the prevention of chronic diseases [3]. Assays of vitamins in blood are carried out as one of several approaches to monitoring of antioxidative defense against different aspects of oxidative damage of important proteins [4]. One recent study [5], for example, found a direct link between "frailty syndrome" in older people and lower vitamin E plasma levels.

Kaempferol is one of the widely studied strong antioxidants. It helps to prevent oxidative damage to cells, lipids and DNA. An *in vitro* study by Kowalski et al. showed that kaempferol inhibited monocyte chemoattractant protein (MCP-1). MCP-1 plays a role in the initial steps of atherosclerotic plaque formation [6]. Kaempferol and other related strong antioxidants can be found in vegetables [7] together with fat-soluble vitamins. However, to the best of our knowledge, a simultaneous analysis of antioxidants, such as kaempferol and fat-soluble vitamins has not been reported yet.

After initial usage of separations by normal-phase chromatography, reversed-phase HPLC has become the prevalent separation technique for the analysis of fat-soluble vitamins. UV spectrophotometry and fluorescence are even today the most frequently applied detection techniques for fat-soluble vitamins [8]. Using a specially designed reverse-phase column, fluorescence and absorbance detection, a laboratory at the US National Institute of Standards and Technology analyzed large number of plasma samples from four studies conducted by the US National Cancer Institute to establish population distribution of blood levels of fat-soluble vitamins and of fat-soluble vitamin related compounds [9]. The mean levels of analytes in blood plasma ranged from 5.9 (vitamin E) to 0.007 μg/ml (*trans*-α-carotene) and all analytes within that range were shown to be detectable either by absorbance or by fluorescence detection. Another study which was published in 2003 reported tocopherol detection limits of 2 ng and 80 pg, respectively, with absorbance and fluorescence detection after a separation on reverse-phase column [10]. Such demonstrated high sensitivity of optical HPLC detection notwithstanding, a need of improved sensitivity for ultra trace levels was noted both in the field of analysis

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Table 1
Comparison of noises at various detection potentials for disposable thin-film carbon and glassy carbon electrodes (number of independent measurement: $n = 3$)

Detection potential (V)	Disposable carbon (pA)	Glassy carbon (pA)
0.50	0.47 ± 0.08	0.54 ± 0.08
0.60	0.66 ± 0.16	0.52 ± 0.05
0.70	0.59 ± 0.04	0.46 ± 0.05
0.80	0.55 ± 0.05	0.48 ± 0.02
0.90	0.70 ± 0.10	0.73 ± 0.26
1.00	0.71 ± 0.15	1.49 ± 0.95
1.10	0.82 ± 0.12	5.12 ± 4.58
1.20	1.58 ± 0.55	12.30 ± 9.46
1.30	2.94 ± 0.40	29.93 ± 10.50
1.40	3.32 ± 0.34	51.77 ± 15.75

of nutrients [11] and for mg amounts of tissue samples [4,12]. One of the methodologies offering a higher sensitivity of detection in comparison with optical techniques is amperometric detection [13].

Since 1980s, there has been a number of publications describing the use of different formats of amperometric detection of fat-soluble vitamins after their separation on various types of reverse-phase columns. Two types of three-electrode cells have been in use for several decades.

The first type is a detection cell consisting, consisting of a glassy carbon working electrode, silver/silver chloride reference electrode and suitable counter electrode. The second type contains a large area carbon electrode, palladium/hydrogen electrode and a graphite counter electrode. The first type is most frequently of thin-layer design. The second type is usually called a Coulometric cell due to the fact that a large area of the working electrode can consume up to 100% of analyte in an electrode reaction producing the detection signal. The potential of hydrogen/palladium electrode is pH dependent (59 mV/pH unit) and is reported to differ by *ca.* 300 mV from that of silver/silver chloride electrode under most common conditions [14]. Another way of estimating the reference potential would be to start with +50 mV [15] at pH 7 and adding 59 mV for each pH unit below that pH (i.e., 296 mV at pH 3 etc.).

In a recent paper [16], detection limits of tocopherols and retinyl acetate with a glassy carbon electrode in a thin-layer cell were reported to be between 140 and 920 pg injected.

Detection limit results reported by the users of large area carbon electrodes in combination with hydrogen/palladium reference electrodes are 20 pg for tocopherols and 5 pg for β-carotene [12].

In this report, we carry out an evaluation of detection limits achievable with glassy carbon electrodes for a broader selection of fat-soluble vitamins and kaempferol as a representative of antioxidants. We also introduce a new type of microfabricated carbon electrodes for thin-layer cells achieving improved sensitivity at relatively high detection potentials required for reproducible detection of fat-soluble vitamins and kaempferol. A single detection potential offering acceptable performance for all analytes included in this study was found.

2. Experimental

2.1. Chemicals

All chemicals for preparation of standards were obtained from Sigma–Aldrich (St. Louis, MO, USA) and used without purification. Methanol was from Burdick & Jackson (B&J Brand High Purity Solvent). Sodium acetate for the preparation of mobile phase was from Dionex (Certified Reagent). Water used for preparation of standards and eluents was of highest available purity (18 MΩ or better).

2.2. Instrumentation for cyclic voltammetry, reverse-phase chromatography and amperometric detection

Dionex ICS3000 liquid chromatograph was used in all experiments described in this report. It consisted of a DP dual gradient pump, an AS autosampler with a cooled sample tray and an electrochemical detector. The data acquisition and system control were by Dionex Chromelon 6.8 software.

Cyclic voltammetry was carried out under static conditions by manually filling a flow through detection cell either with eluent blanks or with samples dissolved in the eluent. The three-electrode detection cell was of thin-layer type [13] and consisted of a suitable working electrode, a silver/silver chloride electrode and a titanium counter electrode. The cell gasket thickness was 25 μm. Cyclic voltammetric scans were carried out by the Chromeleon software and the ICS3000 detector module.

For chromatography, the full ICS3000 system was configured with a Dionex Acclaim 120 C18 column (3 μm packing particle size, 100 mm × 2.1 mm). The elution was either isocratic (Figs. 2 and 3, Tables 1 and 2) or by gradient (Fig. 4). Isocratic mobile phase contained 5 mM sodium acetate buffer pH 5.8 in 94% methanol and 6% water. Gradient elution was carried out by mixing the above acetate buffer with methanol. A detailed description of gradient conditions is given in captions of Fig. 4.

Table 2
Analytical performance comparison of disposable thin-film carbon (A) and non-disposable glassy carbon electrodes (B); detection potentials: +1.30 V

Compound	Type of electrode	Calibration range (nM)	Correlation coefficient	Limit of detection* (nM/pg)
Kaempferol	A	1–1000	0.9996	0.22/0.63
	B	100–10 000	0.9973	1.70/4.86
Retinol	A	LOD–10 000	1.0000	2.04/5.84
	B	100–100 000	0.9997	30.64/87.78
Retinyl acetate	A	LOD–10 000	1.0000	1.80/5.91
	B	100–100 000	0.9980	26.02/85.48
Cholecalciferol	A	LOD–4000	0.9999	7.33/28.19
	B	400–40 000	0.9941	205.72/791.28
γ-Tocopherol	A	LOD–100 000	0.9997	3.74/16.11
	B	100–100 000	0.9994	55.23/237.88
α-Tocopherol	A	LOD–100 000	0.9996	3.90/16.80
	B	100–100 000	0.9992	62.26/268.15

* The limit of detection was calculated as 3 times the noise.