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Quantitative supercritical fluid extractions at trace levels

Sharma, Avadhesh Kumar, Ph.D.

City University of New York, 1990

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QUANTITATIVE SUPERCRITICAL FLUID EXTRACTIONS AT TRACE LEVELS

BY

Avadhesh Kumar Sharma

A dissertation submitted to the graduate faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1990

This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5 July 1990
date

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date

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Abstract

QUANTITATIVE SUPERCRITICAL FLUID EXTRACTIONS AT TRACE LEVELS

By

Avadhesh Kumar Sharma

Advisor : Professor David C. Locke

The technique of supercritical fluid extraction (SCFE) on the analytical scale is explored for the trace level isolation of various organic analytes from tenacious matrices. SCFE yields a clean, rapid, quantitative extract of the analyte which requires no further clean-up prior to analytical determination. Samples weighing a few grams are extracted in a closed system for a period of 15 - 20 minutes at pressures between 4000 and 8000 psi CO_2 and in the temperature range 45 - 65 ^{0}C. To isolate the analyte, the extract-laden CO_2 is depressurized across a short stainless steel tube packed with adsorbent such as silica, C-18, or Celite. The deposited material is eluted with a small volume of organic liquid and analyzed by HPLC, GC, or GC/MS.

The specific methods have been developed for quantitative supercritical fluid extractions to isolate trace levels of anthraquinone from wood and paper pulp, menadione from animal feed, vitamin K-1 from infant food formulas, vitamin A from ready-to-eat breakfast cereals, extraction of 1-nitropyrene from diesel exhaust samples, synthetic mixture of oxy-PAHs from carbon black, nitrosoamines from various cured foods, parathion from oil seeds and grains, and Diazepam from commercial formulations.

The use of highly selective electrochemical detector (ECD) for the HPLC enables direct analysis of extracts with no further clean-up, in the case of vitamin K-1, menadione, vitamin A, parathion, and nitrosoamines, which are extracted along with number of other, non-electoactive substances from lipid rich matrices. The ECD operated in the reductive mode with a silver working electrode offeres high sensitivities (ca. 100 pg) and wide linear dynamic ranges (ca. 10^4) for the analytes investigated.

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I am highly thankful to Professor David C. Locke for his years of guidance, patience, and efforts without which this project would have never been completed.

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No words will ever be sufficient to express my gratitude towards my parents; Prof. O. P. Sharma and Mrs. A. Sharma for their boundless love and sacrifice. It is because of them that I have reached this stage in my life. I will be lucky, if I can ever repay even a tiny part of what I owe to them. Mere thanks would be thoroughly inadequate for the affection I have gotten from my younger brother, Sudhiranjan and sisters, Lata, Shashi, and Manorama. They all were there whenever I needed them.

Last but not the least I must thank my wife Purnima for being so patient with me. Courtesy of the U.S. Immigration Authorities, she is stuck up in N. Delhi.

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INTRODUCTION

INTRODUCTION

The decades of the 1970s and 80s have seen a renewed interest in the field of industrial process- and analytical-scale applications of supercritical fluids along with academic and theoretical research on the underlying fundamental principles of the phenomenon. Every gas possesses a characteristic critical temperature and pressure (T_c and P_c respectively), above which it cannot be liquefied by applying any amount of pressure. At this stage the compound is said to be in its supercritical state (1). Fig. 1 shows the P-T phase diagram of carbon dioxide (CO_2), the substance most commonly used as a supercritical fluid. The critical temperature (T_c) and pressure (P_c) values for CO_2 are 31.1 ^{0}C and 72.9 atm. respectively (2). Above T_c and P_c, CO_2 is in the supercritical state; supercritical fluid extractions and supercritical fluid chromatographic separations are limited to the region above critical temperature and pressure and generally close to the critical point.

Supercritical fluids (SCF) have properties intermediate between those of liquids and gases. The density of an SCF is 2-3 orders of magnitude greater than that of a gas and of the same order of magnitude as that of a liquid (Table 1). This characteristic makes a SCF a dense fluid. The viscosity of a SCF is of the same order of magnitude as that of a gas and is two orders of magnitude smaller than that of a liquid. The diffusivity of an SCF is approximately two orders of magnitude greater than that of a liquid, though much smaller than that of gases. Thus a supercritical fluid could be used as an extracting medium replacing a liquid as a solvent (3).

History

Extractions employing supercritical fluids is not a new idea. The concept of supercritical fluids as solvents has been known to chemical and process engineers for a long time. Hannay and Hogarth first demonstrated at a meeting of Royal Society of London in 1879 that several inorganic salts such as KI, KBr, and $CoCl_2$ could be dissolved in ethanol at temperatures above its T_c (234 ^{0}C) by increasing the pressure, and that the salts could be reprecipitated by decreasing the pressure (6). Supercritical fluid extractions using CO_2 attracted much attention in the latter half of 19th century. Dr. Thomas Andrews, vice-president of Queen's College of Belfast, is credited with establishing the critical values (T_c and P_c) of CO_2 (2). However, it was not until 1960s that the concept of supercritical fluid extractions was utilized for industrial purposes.

Table 1
General Physical Properties of Typical Fluids*

	Diffusivity# (cm^2/sec)	Viscosity (g/cm sec)	Density (g/mL)
Gas 1 atm., ~ 15 -30 ^{0}C	0.1 - 0.4	(1 - 3) x 10^{-4}	(0.6 - 2) x 10^{-3}
Supercritical fluid			
(T_c, P_c)	0.7 x 10^{-3}	(1 - 3) x 10^{-4}	0.2 - 0.5
(T_c, 4P_c)	0.2 x 10^{-3}	(3 - 9) x 10^{-4}	0.4 - 0.9
Liquid (organic solvents, water) ~ 15 -30 ^{0}C	(0.2 - 2) x 10^{-5}	(0.2 - 3) x 10^{-2}	0.6 - 1.6

Self diffusion for gases and supercritical fluids; binary mixture for liquid

* Ref 4

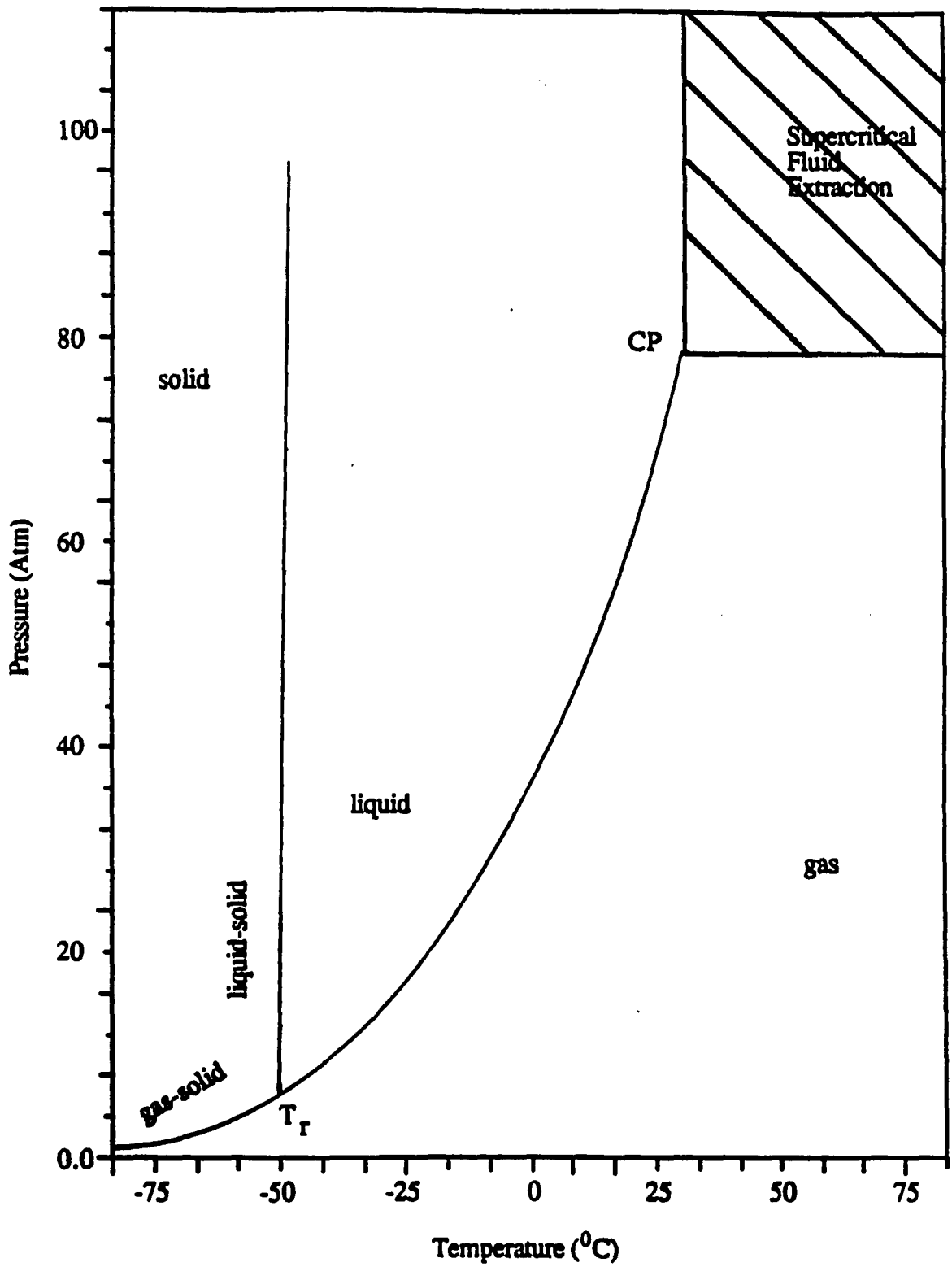

Figure 1. Phase diagram of carbon dioxide (Ref. 1).

Advantages

Extractions performed under supercritical conditions have numerous advantages over classical liquid-liquid and Soxhlet extractions.

Since critical temperatures in the most commonly used gases, CO_2 and N_2O, are relatively low, i.e., a few degrees higher than ambient temperature (Table 2), supercritical fluid extractions are generally performed at moderate temperatures and in the absence of air and light. Thus, thermally labile, photosensitive and easily oxidizable compounds can be safely extracted. The advantage of performing extractions at low temperatures is shared with liquid-liquid extractions but not with Soxhlet extractions, The temperature in Soxhlet extractions rises up to the boiling point of the solvent, which in certain cases can be high enough (such as 80.1 ^{0}C in benzene, 118 ^{0}C in methyl iso-butyl ketone) to cause thermal decomposition. In the case of liquid-liquid and Soxhlet extractions the analyte is usually in contact with light and air, and special arrangements to exclude light and air are difficult, whereas the avoidance of light and air is inherent in the design of supercritical fluid extractors.

The low viscosity of supercritical fluids imparts to them greater powers of penetration into porous solid matrices, thus often resulting in higher recoveries as compared to the extractions using liquids.

The high diffusivity results in faster mass transfer, thus short interphase equilibration times, which in turn means overall faster extractions (a few minutes as compared to hours and days in case of Soxhlet extractions). Consequently, the SCF extractions turn out to be more economic in terms of time as compared to the usual liquid extractions (3).

Pressure-dependent solvent strength is a unique property of a SCF. A supercritical fluid displays a wide spectrum of solvency. The density, hence solvent power of an SCF can be finely tuned by merely changing either the pressure or temperature (4). Thus a single supercritical fluid can function as a multiple solvent system of varying densities. Consequently, simultaneous extraction and fractionation of complex materials can be achieved by changing pressure or temperature, thus avoiding the time-consuming

and painstaking process of fractionation after extraction as usually required in multi-step sample preparation methods.

Removal of solvent from extracted material can be accomplished by simple depressurization of the system, thus bringing SCF to a subcritical state where the solubility of analyte in the gas is small. On the other hand, liquids have to be removed by the time-consuming and less economic process of distillation.

The solvating/displacing properties of an SCF can be dramatically altered by adding polar or non-polar entrainers (usually an organic liquid 2-5%) (5). Knowledge of formation of low boiling azeotropes is necessary in case of Soxhlet extractions, lest a low boiling azeotrope escape without being condensed, thus changing the composition of extracting medium in addition to forfeiting the basic purpose of addition of an entrainer.

Extractions with SCFs are free of complications such as emulsion formation which result in poor recoveries of analyte, or chemical reaction with the matrix or analyte. These possibilities may exist in liquid-liquid and Soxhlet extractions.

Disposal of the solvent in the case of supercritical fluid extractions (SCFE) is trouble-free, at least when CO_2 is used; it is non-toxic and is not a serious environmental hazard. The situation is more serious in case of organic solvents, which in many cases are confirmed/suspected carcinogens, and their disposal has to be in accordance with local, state, and federal environmental protection laws.

A wide range of substances are available that can be used as extraction fluids in SCFE. Some of these are listed in table 2.

Table 2.

Critical Properties of Various Substances*

Solvents	Critical Temperature (°C)	Critical Pressure (atm)
Carbon dioxide	31.1	72.8
Ethane	32.3	42.8
Ethylene	9.3	49.7
Propane	96.7	41.9
Propylene	91.9	45.6
Cyclohexane	280.3	40.2
Isopropanol	235.2	47.0
Benzene	289.0	48.3
Toluene	318.6	40.6
p-Xylene	343.1	34.7
Chlorotrifluoromethane	28.9	38.7
Trichlorofluoromethne	198.1	43.5
Ammonia	132.5	111.3
Water	374.2	217.6
Sulfur dioxide	175.5	77.6
Nitrous oxide	36.5	71.4
Argon	-122.0	48.0
Methane	-82.0	45.8

* Ref. 1

Applications

As pointed out above, extraction utilizing SCFs has been actively pursued on the process scale since the early 1960s, however, the details of many processes are either trade secrets of manufacturers or registered patents of the discoverers, and hence not easily accessible. However, there are a few reviews available on process scale applications of supercritical fluids (1, 4, 7, 8).

The applications of SCFs are numerous and diverse on the industrial scale. Although the focus of this work is quantitative extractions performed at trace levels of organic analytes on the analytical scale, major industrial applications in the fields of food, tobacco, petroleum, pharmaceutical, polymer manufacturing and purification and other miscellaneous applications are briefly mentioned here.

Food

Since CO_2 is non-toxic, inert, and ubiquitous in nature, the Food and Drug Administration has classified CO_2 as Generally Recognized As Safe (GRAS) (9). Hence use of CO_2 for processing of food and natural products meant for human consumption is highly advantageous and desirable. The decaffeination of tea and coffee is the most common and the oldest example of the commercial utilization of SCF CO_2. The usual method is to use organic solvents for the purpose of extraction. In addition to being less economic the process involves handling of large amounts of hazardous organic solvents. In a typical process of decaffeination involving organic solvents, the flavoring substances are removed by means of extraction with petroleum ether from tea or coffee. The deflavorized tea/coffee is moistened and passed through ammonia solution in order to separate the caffeine salts and then the caffeine is extracted by organic solvents such as trichloroethylene or dichloromethane. After the tea or coffee has been dried, the flavor removed earlier is restored. Precautions must be taken to remove all traces of the solvent from tea/coffee (11). A large number patents exist for the decaffeination process using supercritical CO_2 (10, 12). Most commonly, presoaked green coffee beans are extracted with continuously recycled CO_2 at 160-200 atm. The caffeine is extracted from the coffee beans and carried out of the pressure vessel into a washing tower, where it is washed out of CO_2 with water at 70-90 ^{0}C. After 10 hours all of the caffeine is in the wash water, which is then recovered by distillation. The caffeine content of beans is reduced to 0.2%.

Vitzthum and co-workers have patented a number of processes using supercritical CO_2 to prepare "spice extracts" from a number of spices e.g., piperidine from black pepper (4), capsaicine from chilies (13), nutmeg butter from nutmeg (14).

The extraction and deodorization of edible oils is another important area of application of supercritical fluids. Zosel has reported extraction of oil from soybean flakes, and corn using propane, ethane, carbon dioxide, and nitrous oxide (4). Friedrich et al. have reported replacement of hexane with SCF CO_2 for extraction of oil from soybeans (15).

Petroleum Products

Most important area of application of SCFs in the petroleum industry is deasphalting of petroleum. This process has been accomplished by using a number of supercritical fluids such as carbon dioxide (16), methane (17), ethylene (18), ethane, (19) and propane (20). Kerr-McGee developed a supercritical fluid deashing process to separate ash from liquefied coal (21).

Tobacco

Low nicotine or "mild" tobacco has gained popularity because of the toxic effects of nicotine(22). In a typical multistage nicotine removal process, the first step is to isolate flavors from the starting material. The flavors are either isolated as an extract or used to impregnate a previously denicotinized batch by simply allowing the aroma/flavor-laden supercritical stream to expand into the denicotinized batch. The water content of the deflavorized tobacco is increased to about 30%. It has been proven that water aids in the extraction of nicotine. The nicotine is extracted from the deflavorized tobacco at a suitable temperature via an isobaric recycling operation. Selective adsorbents are used to isolate nicotine from the supercritical fluid.Finally, the aroma/flavor is restored to the denicotinized batch. The nicotine content of tobacco is reduced by 94.7%. The tobacco denicotinized using supercritical fluids has all the flavor and texture of natural tobacco (23).

Pharmaceuticals

A number of pharmaceutically important compounds have been extracted using supercritical fluids. Krukonis etal.have isolated potential antineoplastic agents such as maytensine from plants. Supercritical carbon dioxide and ethylene were used for this extraction work (24).

Other examples worth mentioning are extraction of crude opium for codeine, narcotine and papaverine (25, 26, 27), vitamin-oil mixtures for cholesterol, vitamins D_3, K_3, A and E (28, 29), and cinchona bark for quinine (25, 26).

Organics from hazardous waste

Major areas of interest include : regeneration of adsorbents such as activated charcoal, oxidation of hazardous waste, extraction of organics from aqueous streams, leaching of contaminated soil samples (30).

Polymers

Polymerization of ethylene has been achieved at a pressure of 40,000 psi and 250 ^{0}C, in supercritical ethylene in the presence of a free radical initiator such as O_2 or a peroxide. The polymer is recovered at a pressure of 4000 psi. (31, 32). Low molecular weight cyclic oligomers have been recovered from polyoxyalkylene by bubbling supercritical propylene at 100 ^{0}C and 80 atm. (33). Specialty materials like silicone oils, polycarbosilanes, polyhalocarbons which are used as lubricants, hydraulic fluids, and surfactants usually require high purity and narrow molecular weight ranges. Highly satisfactory results have been reported for purification of these materials using supercritical fluids (1, 34).

Chemical reactions

The major advantage of performing reactions in supercritical media is that the reaction products can be precipitated as the reaction proceeds, thus avoiding unwanted side reactions. For example, Alexander and Paulaitis carried out a Diels-Alder reaction of isoprene and maleic anhydride at very low reactant concentrations in supercritical CO_2 (35). Koll and Metzger have reported thermal degradation of cellulose in supercritical acetone at considerably lower temperatures, thus improving the yield and product distribution (36).

Analytical applications

The application of supercritical fluid technology on the analytical scale for quantitative extraction of trace analytes is relatively new. The technique can be used either as an off-line or an on-line technique. In the first case, the analyte is extracted, collected and later on identified and quantified by various chromatographic and spectroscopic methods in two different steps. In the case of on-line methods, the analyte is extracted , trapped and analyzed sequentially, without analyte ever leaving the system. Further, the extractions

ever leaving the system. Further, the extractions could be carried out in either a dynamic mode, where a constant flow of the supercritical fluid is maintained or a static mode, where the extraction is carried out in a closed system.

Various techniques of interfacing a SCFE system with an SFC (supercitical fluid chromatograph) and a GC have been reviewed by Raynor and co-workers (37). Unger and Roumeliotis have described in detail the interface of SCFE with HPLC and have reported continuous extraction of valtrate and didrovaltrate from *Radix valerianae* with CO_2 at 313 K and 96 bar (38). Gmuer, Bosset and Plattner have reported the construction of a prototype for a direct SCFE and SFC interface and have reported the analyses of some natural substances such as cheese, butter, coffee, tobacco, and camomile (39).
Hawthorne and Miller have reported on-line continuous extraction of polycyclic aromatic hydrocarbons (PAHs) from NIST standard reference material (SRM) # 1650. The extraction was carried out at 300 atm. for 90 min. at 45 ^{0}C. Recoveries comparable to Soxhlet extraction have been reported for fluoranthane, pyrene, benzo[a]pyrene and benzo[a]anthracene at 300 atm. in just 30 min. when 5% methanol was used as an entrainer. Class selective extraction of an NBS 1650 sample at 75 atm., 45 ^{0}C, for 5 min. followed by more rigorous extraction at 300 atm., 45^0 C, for 90 min. yielded a very good separation of alkanes and PAHs. Approximately 85% alkanes were separated in the first fraction and 90% PAHs were identified in the second extract (40). Hawthorne and Miller have reported on-line SCFE-GC of PAHs and PCBs, from NBS sample SRM# 1649 using N_2O at 300 atm. and 45 ^{0}C for a period of 20 min. (41). Recovery of PAHs in supercritical N_2O with 5% methanol, N_2O alone, and ethane has been compared. N_2O with 5% methanol was best of the three and gave recoveries comparable with an 8 h Soxhlet extraction or a 4 h sonication with either benzene or methylene chloride (42).
A supercritical fluid CO_2 extractor coupled with capillary GC has been used for analysis of flavor and fragrance compounds in rosemary, thyme, cinnamon, spearmint flavor chewing gum, orange peel and aromatic cedar wood. The time of extraction is usually 10 min. or less. The extract is cryogenically focussed on the GC column (43). Using SCFE-GC, Hawthorne, Miller, and Kreiger have reported the presence of a variety of organic compounds in various products: PAHs from treated wood, urban dust, and river sediments, phenolic substances from wood smoke particulates, nicotine from tobacco, biological markers from coal, and flavor components from food products (44). Quantitative extractions on an analytical scale of PCBs, PAHs, hetro atom

PAHs, and n-alkanes from polyurethane foam sorbent plugs have been reported by Hawthorne etal. using supercritical fluid CO_2 (45). Hawthorne and co-workers used an LC pump to generate supercritical fluids. The extraction chamber was fabricated using stainless steel fittings and had an internal volume of 0.1 mL. A stainless steel frit was placed inside the fitting to prevent the sample from plugging the outlet restrictor (Fig 2). Supercritical pressures were maintained inside the extraction cell using a fused silica capillary tubing (40-45). The system designed by Hawthorne and co-workers suffers from a number of drawbacks. Since an HPLC syringe pump is used for generating supercritical fluids, the pump has to be filled with liquid CO_2 or N_2O and the pump head, gas cylinder (with a dip tube), delivery lines etc. have to be kept at low temperatures. Further, the time of extraction is limited by the volume of the pump cylinder. The internal volume is low (1 mL), thus limiting the size of sample that can be extracted. This difficulty was encountered by authors themselves when they had to design another extraction chamber for a 500 mg sample size (40). To regulate the out-flow of the supercritical fluid a silica capillary tube is used, which is fragile and is easily contaminated. A high precision valve could be used instead to regulate the flow rate of the exiting fluids. Last but not the least, even though authors report quantitative exractions (41, 43), the absence of optimization of extraction conditions of target analytes with respect to parameters such as equilibration time, pressure and temperature is obvious.

Smith and Wright have reported on-line SCFE-CI mass spectrometry for rapid identification of parts per trillion levels of several trichothecene mycotoxins from a complex matrix such as wheat (46). Wright etal. have reported the extraction of organic compounds like coronene, chrysene, ruberene, 1-nitropyrene, dibenzo[a,i]carbozole, benzanthracene etc. from various adsorbents and particulate matrices. The extraction from these matrices was studied in three different supercritical fluids, CO_2, CO_2+20% methanol, and isobutane. It was found that high molecular weight and less polar substances were better extracted with isobutane, whereas CO_2 and methanol was better choice for polar compounds (47). Wright and co-workers have reported an on-line SCFE-GC, a fully automated system of sample preparation and analysis. The authors demonstrated that high molecular weight PAHs are extracted quantitatively at high pressures, when the density of CO_2 is high and low molecular weight PAHs are extracted at low pressures, when the density of CO_2 is relatively low (48). Wright and co-workers also used a syringe pump (Varian, model 8500) to generate supercritical pressures (Fig. 3).

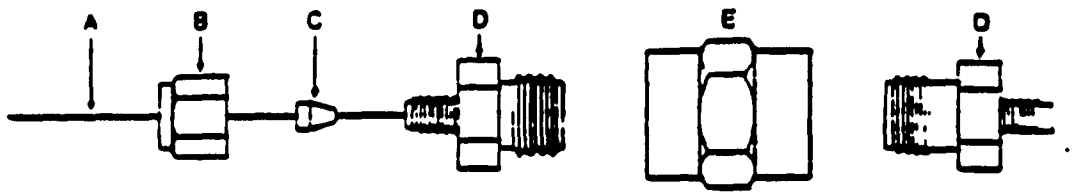

Fig. 2. Components of the supercritical fluid extraction cell (Hawthorne et al., Ref. 40-45). The components are (A) 10-cm x 25-μm i.d. fused silica capillary, (B) 1/16" nut, (C) 15% graphite, 85% polyimide ferrule, (D) 1/16" (tubing fitting) x 3/16" (NPT thread) union, and (E) 3/16" x 3/16" (NPT thread) female union.

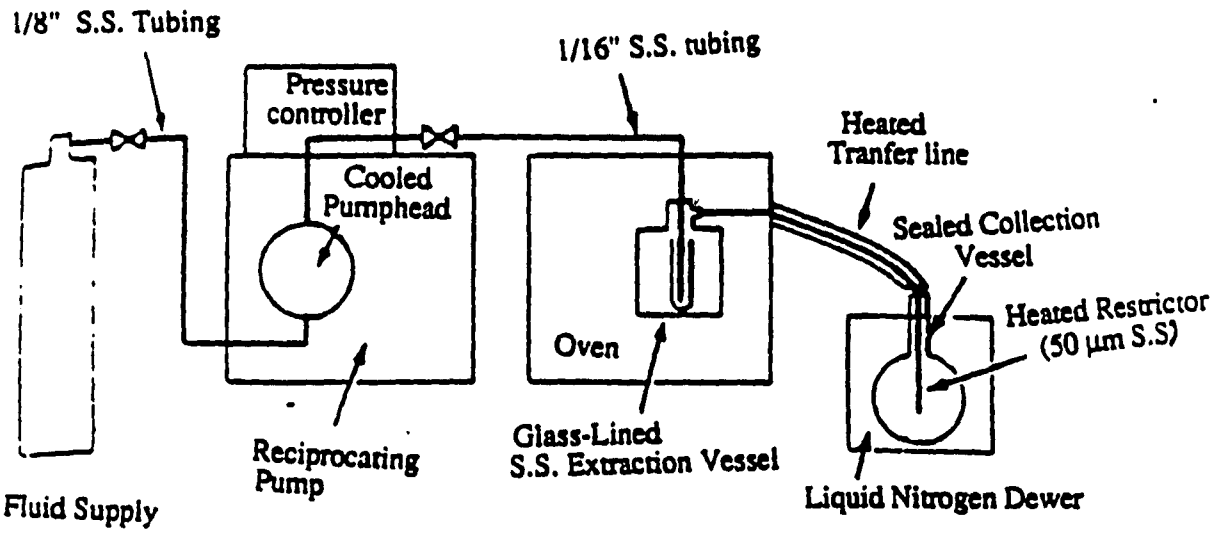

Fig 3. Schematic of supercritical fluid extraction apparatus (Wright et al., Ref. 48)

Thus, this system also suffers from the shortcomings listed above, and would not be able to pump CO_2 or N_2O directly into the extraction aasembly. The stainless steel extraction chambers fabricated by using Swagelok fittings and a stainless steel spacer with a volume of 15 μL are suited only for extremely small sample sizes (such as 10 mg) (48). Another variation of extraction chamber constructed from 316 stainless steel high pressure vessel (Fig. 4) provides safe operation at pressures over 400 atmospheres and temperatures greater than 200 ^{0}C. The transfer line from the pump extends to the bottom of the extraction chamber where the sample is placed in a borosilicate glass tube. The extraction cell body and the top are sealed with double teflon O-rings. The major advantage of this design is that it provides efficient contact of the matrix and the supercritical fluid, by allowing the supercritical fluid to move from bottom to top before exiting the system (47). A capillary restrictor was used to regulate the flow of supercritical fluids exiting the system and suffers from the problems of fragility and cross contamination.(46-47).

Supercritical CO_2 has been applied by Locke, Scott and co-workers for the extraction of PAHs from urban aerosols and fly ash samples at pressures of 4000-5000 psi, 45 ^{0}C, for 30 min. equlibration. Waters C_{18} Sep-Paks were used as collectors. The authors were able to identify and quantify a large number of PAHs such as phenanthracene, anthracene, fluoranthene, chrysene, benz[a]anthracene, benz[b]fluoranthene, benz[a]pyrene, dibenzo[a, h]anthracene, benzo(ghi)perylene, etc. Comparable or better recoveries of the above mentioned PAHs as compared to Soxhlet extraction for 8h with cyclohexane were reported (49, 50).

Schantz and Chesler have reported higher recoveries in nearly 4h, and at 40 ^{0}C and 345 bar pressure for a number of PAHs from urban particulate sample (NBS SRM 1649) and PCBs from sediment, when compared with classical Soxhlet extraction with methylene chloride for 16 h. The recoveries were in excellent agreement with certified values and SCFE proved to be more efficient than Soxhlet extraction in case of benzo[ghi]perylene and indeno [1, 2, 3,-cd]pyrene (51).

Sugiyama and Saito developed a double stage separation and analysis method incorporating supercitical fluid extraction as the first step and supercritical fluid chromatography as the second separation step. Directly-coupled SCFE-SFC, monitored with a highly sensitive multiwavelength detector, was performed on powdered coffee beans and separation was carried out without any special pretreatment. This detector

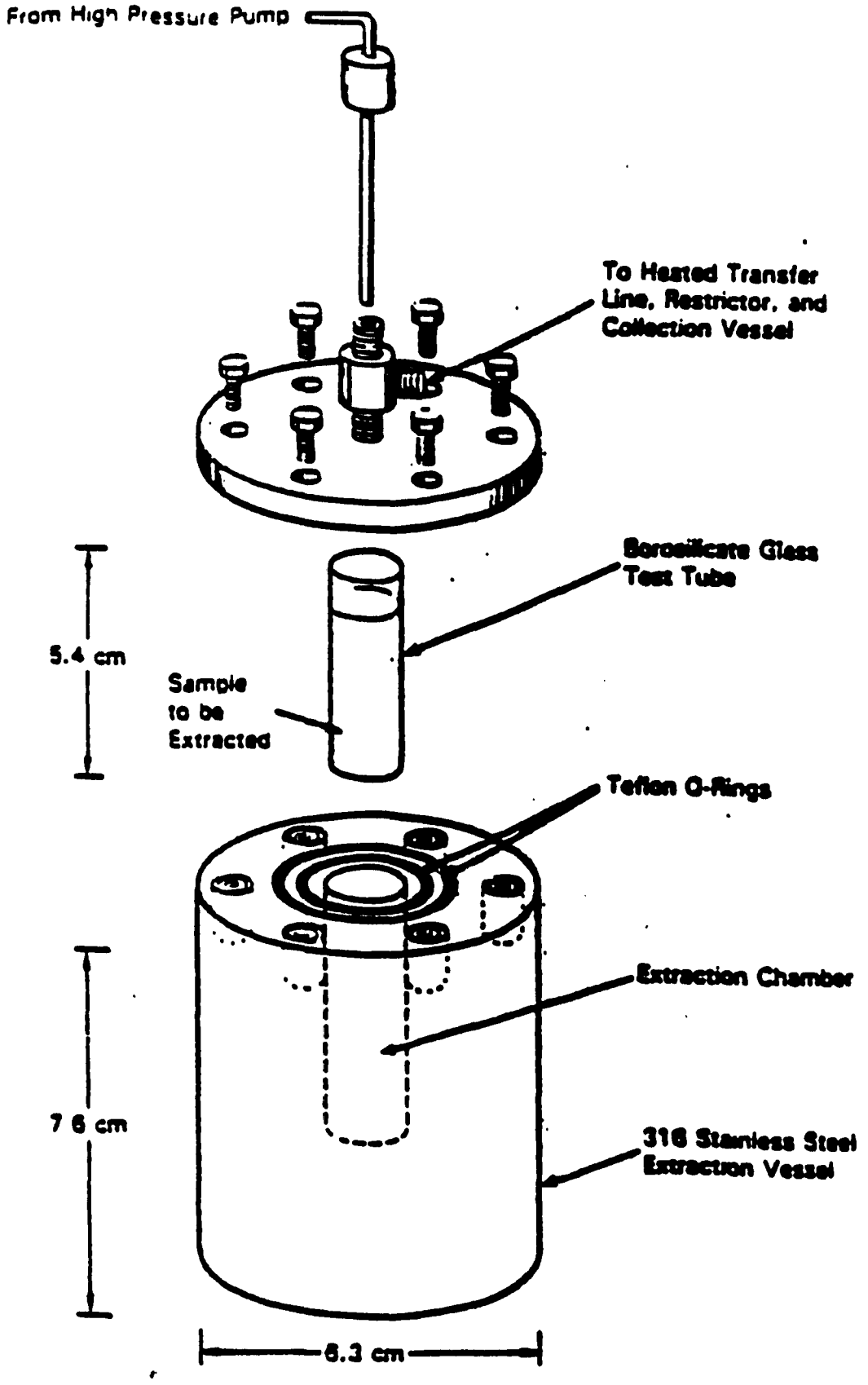

Fig. 4. Design of supercritical fluid extraction vessel (Wright et. al., Ref. 47).

permits on-line UV spectrum monitoring of the extraction process, therefore, optimal extraction parameters can be investigated at low cost without operating a pilot-plant extraction system which requires large amounts of samples and extraction medium (52). Sugiyama and Saito also isolated thermally unstable lemon peel oil with supercritical carbon dioxide extractions performed at various temperatures and pressures. By changing the temperature and pressure the authors were able to demonstrate the variation in strength of supercritical fluids. An empty HPLC column (35 mm x 4.6 mm I. D.) was used as extraction chamber along with a capillary restrictor (53).

McNally and Wheeler have reported SCFE-SFC of two herbicides, diuron and linuron and their principle metabolite, 3,4, dichloroaniline, from sandy and loam soils. The optimization of extraction conditions and the effect of polar entrainers was studied. It was found that high temperatures and high pressures resulted in better extraction effficiences and polar modifiers also increased the effficiency of extraction. The whole process did not take more than 1 h of sample handling as compared to the classical Soxhlet extraction which takes about 1 day (54).

Campbell and Lee utilized the fact that the density and hence solvent strength of a supercritical fluid could be finely tuned by simple pressure programming. A solvent refined coal heavy distillate and a crude oil were fractionated (using supercritical fluid extractions employing CO_2) on columns packed with silica according to the number of aromatic rings. Low molecular weight PAHs came out first, at low pressures, and more complex high molecular weight PAHs came out much later, at high pressures when density was quite high (55).

Supercritical CO_2 has been successfully employed to remove the adsorbed organic pollutants from Tenax-GC traps. The process of desorption by supercritical CO_2 is far superior to the conventional thermal desorption methods. To demonstrate the universality of application of CO_2, four different compounds representing different chemical classes were selected: hexachlorocyclohexane (chlorinated hydrocarbon), hexachlorobiphenyl (PCB), anthracene (PAH), and Parathion (organophosphate pesticide). All compounds are environmentally significant. In the case of hexachlorobiphenyl and Parathion, the thermal desorption method gave only 13% recovery as compared to supercritical CO_2 which gave recoveries for all the four above mentioned compounds in excess of 90% (56). The same work was repeated using four different polyimide

sorbents in place of Tenax-GC. Again, recoveries using supercritical carbon dioxide were in excess of 90% for all the compounds chosen, although the process of desorption required longer time as compared to Tenax-GC (57).

Using SCFE-SFC, Taylor and Ashraf-Khorassani extracted and separated di-n-propyl adiapate, triacetin, 2-nitrodiphenylamine, and nitroglycerin from a double-based propellant. Conventional Soxhlet extraction was not able to extract the minor components, presumably responsible for rendering the propellant useless. On-line FTIR was used for identification (58). Using a specially designed extractor, the authors have recently reported quantitative supercritical fluid extraction follwed by supercritical fluid chromatography of diisopropyl methylphosphonate (DIMP) in water (59).

Ramsey and co-workers reported SCFE/SFC of a group of veterinary drugs including trimethoprim, hexestrol, diethylstilbestrol, and dienestrol and analyzed using SFE-SFC coupled to MS-MS from a freeze dried sample of pig kidney (60).

Chapter 2

SUPERCRITICAL FLUID EXTRACTION APPARATUS AND GENERAL EXTRACTION PROCEDURE

APPARATUS

Fig. 1 shows a schematic diagram of the supercritical fluid extraction apparatus. The carbon dioxide (Linde, bone dry grade) gas supplied through a single stage tank regulator was filtered through an Autoclave Engineers (AE) 5 micron cup type line filter (AE model # CXF 4-5). The system was then pressurized by a motor-driven, single ended diaphragm compressor (American Instrument Company model #J 46-13411). From this point, the apparatus was thermostated in a Lab Line Instruments Imperial II circulating heat oven, which has temperature control to $\pm$ 1^{0}C. All 1/4" connecting tubes (AE model # 15-081), valves (AE 20-SV 4081) and attachments (tee AE model # CTX 4440) were of 316 stainless steel, capable of withstanding pressures up to 1360 atm. A rupture disc rated at 1160 atm. was incorporated into the system to prevent accidental over-pressurization. The extraction chamber was a 3/8" o.d. x 6" long s.s. tube. The extract was collected on a stainless steel tube, 1/4" o.d. x 5" long, which was connected to the low pressure end of the system via 1/4" elbow. The stainless steel tube acts as a trap and is usually packed with common adsorbents such as silica, Tenax, or alumina.

PROCEDURE OF EXTRACTION

To perform a supercritical fluid extraction, the extraction chamber was disassembled and cleaned with acetone and cyclohexane and dried. In the case of liquid samples, a known volume of the sample was adsorbed onto a solid support such as Chromosorb W or streaked onto glass fiber filter paper and placed into the extraction chamber. In the case of solid samples, a known weight of the solid was placed into the extraction chamber. Glass wool was placed into both ends of the extraction chamber to prevent movement of the sample. The extraction train was reassembled. With all the valves open, carbon dioxide under low pressure was allowed to flow through the system, to remove any air present. The valves were then closed. The system before the extraction chamber was pressurized with carbon dioxide to the desired value. The oven was also turned on to attain the desired temperature. The valve before extraction chamber was opened and system was repressurized to the desired value. The temperature and pressure were stabilized, the conditions of temperature and pressure were maintained for a certain period of time (time of equilibration). At the end of equilibration time, the compressor was turned off and the valve after the chamber was carefully partially opened. The flow of carbon dioxide exiting the system was maintained at approximately 100 mL/min. The supercritical carbon dioxide

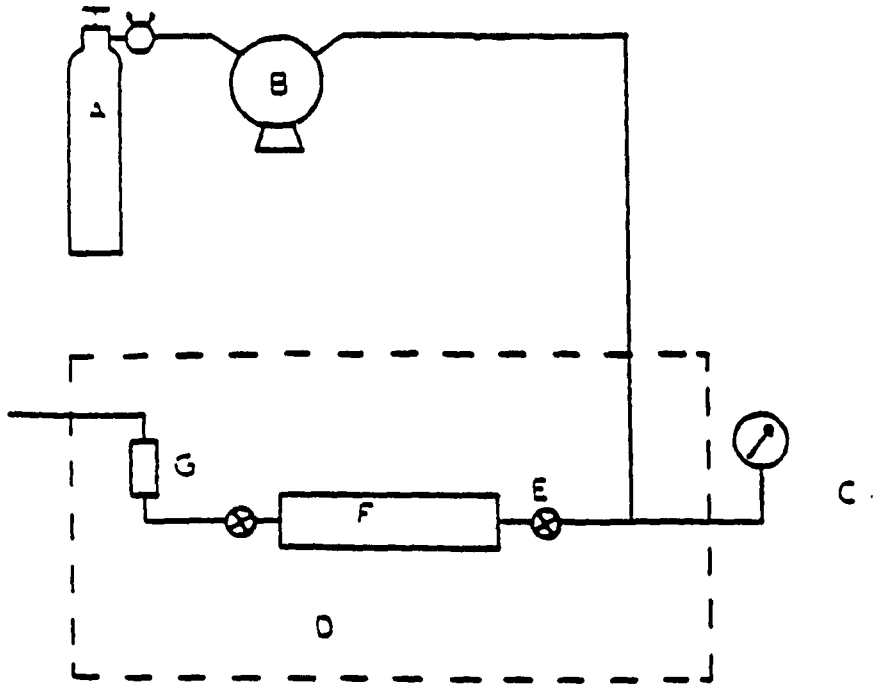

Fig.1 Supercritical fluid extractor. A=carbon dioxide cylinder with high-pressure regulator; B=diaphragm compressor; C=pressure gauge; D=drying oven; E=valve; F=extraction chamber; G=collector-trap.

containing the extract passed through the second valve and the system was depressurized. The extract was collected on the trap packed with trapping materials such as silica gel or Tenax. The trap was eluted with 5-10 mL of organic solvent like dichloromethane or acetone. The organic solvent was rotovaporised to dryness and the extract was reconstituted in either in 1 mL of HPLC mobile phase for HPLC analysis or 1 mL of dichloromethane for GC or GC-MS analysis.

The methodology thus far developed is an off-line technique. It has a number of distinct advantages when compared with the ones currently adopted by Hawthorne et. al and Smith et. al. (Chapter 1). The pump used in this apparatus is capable of compressiing gases as such, unlike the normally-used syringe-type HPLC pumps where the pump head, delivery lines, cylinder head (equipped with a dip tube) have to be cooled to keep commonly used gases such as CO_2 and N_2O in the liquid state in order they could be pumped. Moreover, the continuous supply of the supercritical fluid does not depend on the volume of the solvent reservoir as is the case with most syringe-type pumps; the gases are compressed directly from the cylinder ensuring a continuous supply of the supercritical fluid for a long period of time (c.f. Hawthorne et. al. and Smith et. al., Chapter 1). The internal volume of the extraction chamber is approximately 11 mL; hence it is suitable for large sample sizes (such as 1-2 g) as well. Instead of a silica capillary restrictor, a stainless valve is used to maintain supercritical pressures inside the system. The disadvantages of fragility and cross-contamination associated with capillaries are totally eliminated. Even though an off-line methodology, it has a distinct advantage over the on-line methods where an extraction system is interfaced with a detection system. In on-line method the choice of detection is limited to the one which is interfaced, on the other hand in the present off-line method, the choice of detection methods is diverse i.e, HPLC-ECD, HPLC-UVD, GC-FID, GC-electron capture detector, GC/MS etc. Thus, more than one off-line method colud be applied to a sample too.

Despite of all the advantages listed above, the system needs some modification/improvements. First, instead of using the Swagelock unions to connect the extraction chamber to the 1/4" unthreaded tubing, threaded unions and threaded nipples manufactured by Autoclave Engineers, Inc. (AE) should be used. The unions and nipples made by AE are more rugged and have a longer life time. Moreover, in event of overpressurization or wearing out of threads and ferrules in connecting unions or tubes, the system would leak first rather causing a catastrophic depressurization. In order to run a continuous extraction and/or let the fluid escape at a certain rate the on and off valves as used in thepresent system would be of little use; a high pressure metering valves

(AE # 60 VRM 4882) could be used instead. Last but not least, instead of a safety rupture disc, a safety valve set at 11000 psi (AE # 20-RUP 9072) could be used. The advantage is that in event of accidental over-pressurization, the excess of fluid will escape through a drain tube provided for this purpose and the system along with safety valve is ready to be used again, whereas in case of a safety disc being ruptured once, it has to be replaced with a new one.

In the following chapters quantitative supercritical fluid extractions (i.e., extraction efficiency of more than 90%) to isolate trace levels of anthraquinone from wood and paper pulp, menadione from animal feed, vitamin K_1 from powdered infant formulas, vitamin A from ready-to-eat breakfast foods, from infant formulas, diazepam from Valium tablets, nitrosoamines from various food products, parathion from oil seeds and grains, simultaneous extraction and fractionation of 1-nitropyrene from diesel exhaust particulates (NBS SRM 1650), oxy-PAHs from a synthetic mixture are presented. The chromatographic separation and identification of the extract was done by using GC-FID, GC-MS, and a lab-fabricated HPLC-ECD. The highly sensitive and selective electrochemical detector (ECD) for HPLC was used in both reductive and oxidative modes.

The details of chromatographic separations and determinations of each of above listed analytes are described in their respective chapters.

Chapter 3

DETERMINATION OF ANTHRAQUINONE IN PAPER AND WOOD

INTRODUCTION

Anthraquinone is used in catalytic amounts in wood pulping operations to aid delignification and to increase pulp yield (1). Methods for the analysis of wood and paper generally involve Soxhlet extraction with chloroform or dichloromethane followed by high-performance liquid chromatography (HPLC) with UV spectrophotometric detection (2), or on-line post-column reduction and UV detection (3); straight electrochemistry (without HPLC) using either chemical reduction of anthraquinone and electrochemical oxidation of the product (4), or differential pulse polarography (5); or gas chromatography (GC) and GC-mass spectrometry (MS), (6, 7). The isolation step requires a time-consuming Soxhlet extraction, or multiple batch solvent extractions. The latter suffers from formation of emulsions, often resulting in low recoveries (2,8). Nelson and Citetek (8) avoided these problems by using a Waters C_{18} Sep-Pak to isolate anthraquinone from black liquor, followed by HPLC-UV. UV detection offers little selectivity, responding to anthraquinone and to other compounds co-extracted with it. The limit of detection is 0.2 μg (8).

The extraction of anthraquinone from Kraft paper and pine plywood fortified with anthraquinone using supercritical fluid CO_2 is reported here. Anthraquinone is determined in the extract using HPLC with electrochemical detection at a silver electrode. This method is rapid, highly selective and sensitive to subnanogram levels of anthraquinone in paper and wood.

EXPERIMENTAL

Chemicals

9,10-Anthraquinone (Sigma) was used without further purification since HPLC and GC-MS showed essentially one peak. Acetonitrile, methylene chloride, and acetone were HPLC grade. The HPLC eluent was 80:20 (v/v) acetonitrile- aqueous pH 6.8 buffer (0.025 M each KH_2PO_4 and Na_2HPO_4). CO_2 was bone-dry grade(Linde, Long Island City, NY).

Supercritical fluid extraction

The supercritical fluid extraction apparatus and detailed procedure were described in chapter 2. To carry out an extraction, about 6 strips (3 in x 1/2 in, accurately weighed) of Kraft paper previously soaked and dried, or approximately 0.5 g of pine plywood sawdust were packed into the chamber and held in place with glass wool. The paper and sawdust samples were fortified with anthraquinone deposited from a stock solution in methylene chloride and dried before being loaded into the chamber. The extraction chamber was assembled into the extraction train, brought to 65 ^{0}C, flushed briefly with atmospheric pressure CO_2, the exit valve closed, and the sample pressurized to 8000 psi and equilibrated for 20 min. The exit valve was then carefully opened to bleed out the extract-laden CO_2, and the extract collected on the silica gel trap. The silica trap was washed with 15 mL methylene chloride-acetone (50:50), the solution evaporated in a rotary evaporator to dryness, and the residue reconstituted in 10 mL of mobile phase for HPLC.

HPLC

A chromatograph was assembled from a Varian 8500 syringe pump, a Rheodyne 7125 sampling valve with a pneumatic sample degasser and loading device and a 20-µl sample loop, and a lab-built electrochemical detector (Appendix 1). The column was 150 x 35 mm I.D., packed with 10 µm µBondapak C_{18} (Waters Assoc.). The eluent was degassed by bubbling extensively with helium. The injector, column, and detector were compactly assembled in a 9 in. x 9 in. x 6 in. Lucite box, the walls of which held the PC boards of the potentiostat and the electronic controls.

GC-MS

For comparison with the HPLC-ECD, some extracts were analyzed by GC-quadrupole MS using a Hewlett-Packard 5988 A system with a Hewlett-Packard 1000 data system. A 30 m x 0.025 mm I.D. DB-5 fused-silica capillary column (J & W Scientific) was used. The initial temperature, 150 ^{0}C, was held for two 2 min followed by temperature programming at 10 ^{0}C/min to 250 ^{0}C where it was held 20 min.

RESULTS AND DISCUSSION

Hydrodynamic voltammogram

Fig. 1 shows the hydrodynamic voltammogram of anthraquinone on the silver electrode. The peak current reaches a plateau at about -1.1 V vs. calomel, which was the potential used in all subsequent experiments. The difference in the value of the applied potential at 75% of the plateau current and that at 25% of the plateau current is 82 mV, indicating the reduction of anthraquinone under these conditions is not fully reversible. Reversibility is inferred from a value of 28.2 mV for the potential difference at these two currents, for a convective diffusion-controlled 2-electron process (9).

Effect of flow rate on response

As indicated in Fig. 2, at effluent flow rates up to about 100 mL/h, the peak current increases approximately with the 1/3 power of the flow-rate. This suggests that the reduction current is controlled by convective mass transport (10). Since the response becomes independent of flowrate at higher flows, 2 ml/min was used in subsequent experiments.

Response to anthraquinone

A series of standards in concentration range from 0.114 mg/ml to 0.0012 mg/ml was prepared in the mobile phase by serial dilution. The resulting peak currents at -1.1 V vs calomel and 2 ml/min are plotted vs weight injected in Fig. 3. The response is a linear function of weight injected, with a slope of 1.0, over at least 4 orders of magnitude of sample weight (Fig. 4). The noise level was 300 pA, so that a at signal-to-noise ratio of 3, the minimum detectable quantity is 210 pg.

SCFE efficiency

Ability of supercritical-fluid CO_2 to extract anthraquinone was assessed by spiking 300 mg of Kraft paper strips (3 in. x 1/2 in) and 500 mg sawdust samples with levels of anthraquinone varying from 5 μg to 100 μg for the paper and 3 μg to 500 μg for

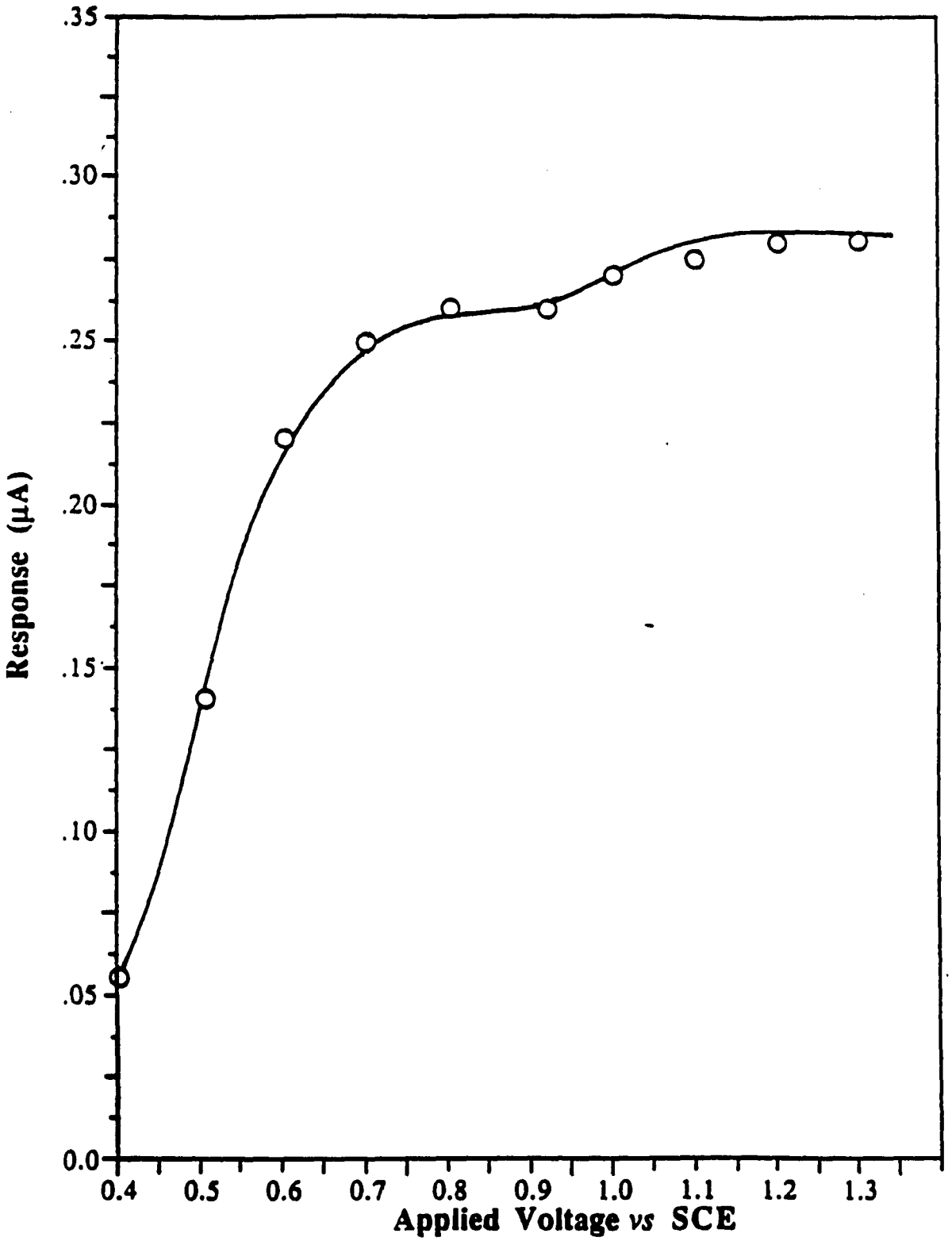

Figure 1. Hydrodynamic voltammogram of anthraquinone on silver electrode vs. SCE. Column, 150 x 3.9 mm I.D. μBondapak C-18 (10 μm); Mobile Phase, 80:20 (v/v) acetonitrile-aqueous 0.05 M phosphate buffer (pH 6.8); Flow rate, 2 mL/min. Sample size, 0.44 μg anthraquinone per 20 μl mobile phase injected at each potential setting.

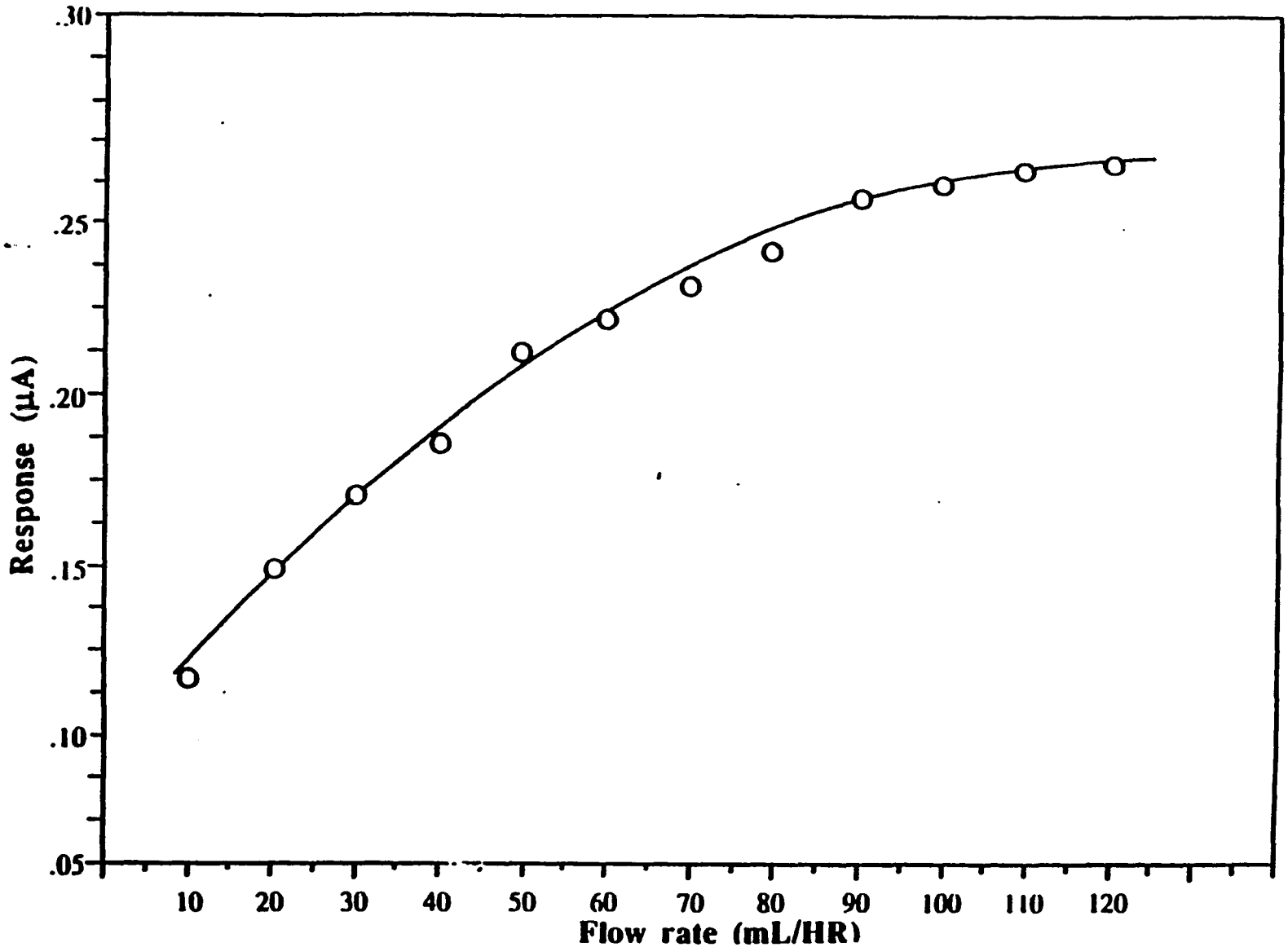

Figure 2. Effect of flow rate on response to anthraquinone. Applied potential, -1.1 V vs SCE; Chromatographic parameters (except flow rate) same as in Figure 1. Equation of the curve is $I_p = F^{0.352}$

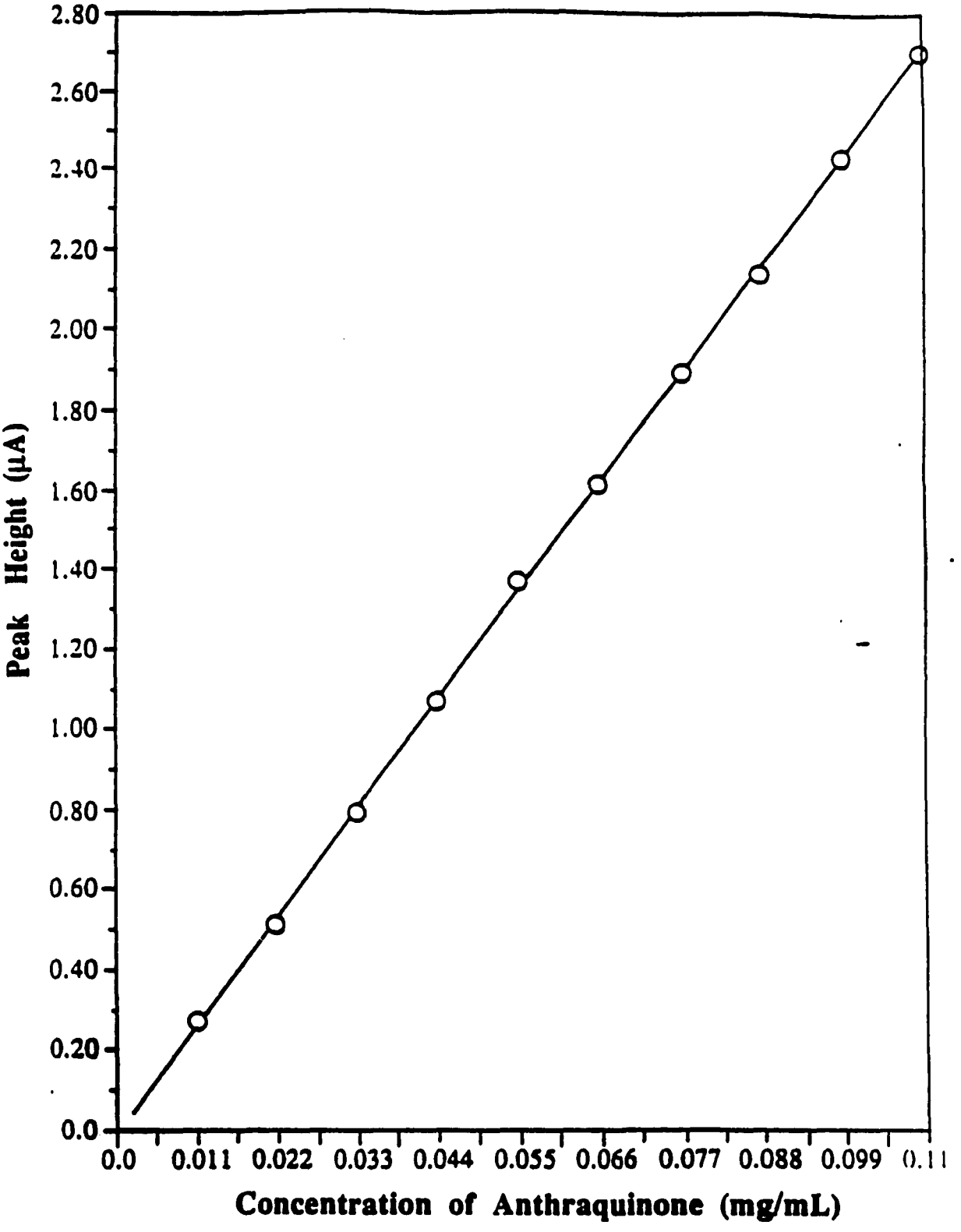

Figure 3. Calibration curve for anthraquinone obtained from 20 µl injections onto a 150 by 3.9 mm ID µBondapak C-18 column with a acetonitrile-(pH 6.8) phosphate (80+20, v/v) mobile phase. Flow rate was 2.0 mL/min. Potential of silver working electrode was -1.1 V vs SCE.

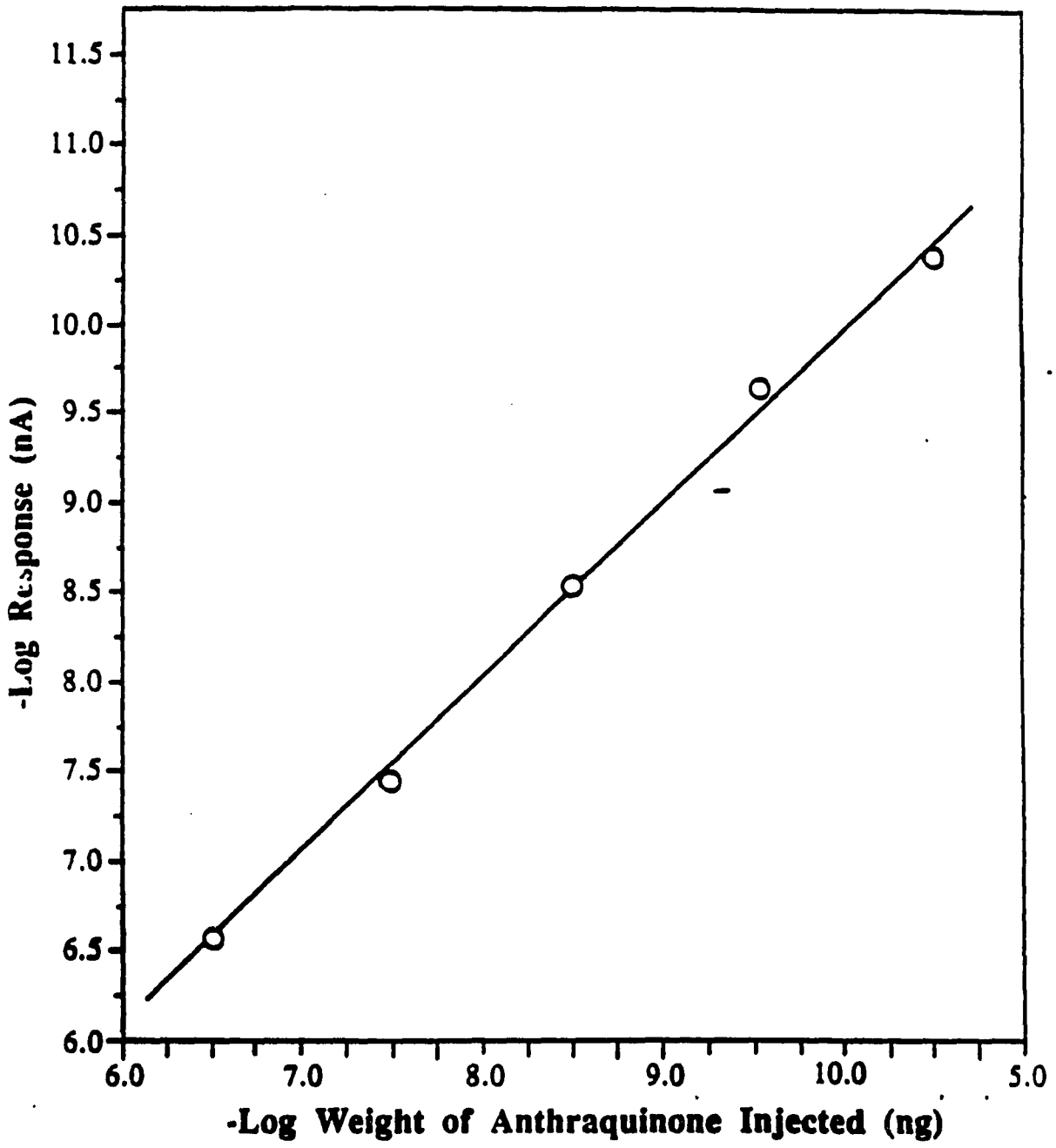

Figure 4. Linearity of detector response to amounts of anthraquinone in 20 µl samples. Chromatographic conditions: column, 150 x 3.9 mm ID µBondapak C-18; eluent, 80/20 acetonitrile -0.05M phosphate buffer (pH 6.8); flow rate, 2.0 mL/min; applied potential of working electrode, -1.2 V *vs* SCE.

TABLE 1
SUPERCRITICAL FLUID EXTRACTION RECOVERY OF ANTHRAQUINONE FROM PAPER AND SAWDUST

Kraft paper		Sawdust	
Weight added (mg)	Recovery (%)	Weight added (mg)	Recovery (%)
1.0	100	0.5	94
0.75	98	0.25	96
0.50	96	0.10	90
0.40	96	0.05	91
0.25	100	0.01	83
0.20	93	0.005	76
0.10	88	0.003	68
0.05	92	---	---

the sawdust. As shown in table 1, good recoveries from the paper were obtained down to lowest level studied. In the case of sawdust, however, the percentage extracted under the conditions used falls off below about 10 μg anthraquinone, presumably because it is strongly adsorbed to some active site on the wood or anthraquinone may get reduced to hydroxy quinone in the matrix. The efficiency of extraction in such cases could be improved by addition of polar entrainers such as methanol to the sample matrix. It is clear from the data that approximately 1 μg is effectively irreversibly adsorbed, which accounts for the percentage extracted value.

Analytical Precision

The daily repeatability of the method was evaluated for the percentage recovery from the sawdust samples. The average precision (coefficient of variation) for the three extractions at each level of spiking was 2.2%, which was approximately independent of the level of anthraquinone added. The day-to-day precision was determined by extracting sawdust spiked at five different levels (2 mg to 10 mg) on each of the five consecutive days. The average recovery was 93.2% with an average coefficient of variation of 3.8%, again almost independent of anthraquinone added.

COMPARISION WITH SOXHLET EXTRACTION

One of the advantages of SCFE is speed; a 20 min extraction gives 90% recovery. Soxhlet extraction of spiked Kraft paper samples with acetonitrile or methanol, however, required at least 4 h to reach 82% recovery. After 1 h, 16% was extracted, after 2 h, 39%, 3 h, 61%, and 4 h, 83%. In addition, there are no emulsions or other technical difficulties with supercritical fluid extraction, no light or air; and fewer impurities in CO_2 than in ordinary solvents.

COMPARISION OF HPLC-ECD WITH GC-MS

A typical HPLC-ECD chromatogram is shown in Fig. 5; only one peak is observed in a de-aerated sample. On the other hand, the mass spectrometer is a universal detector, and a fairly rich chromatogram results from SCFE of a spiked

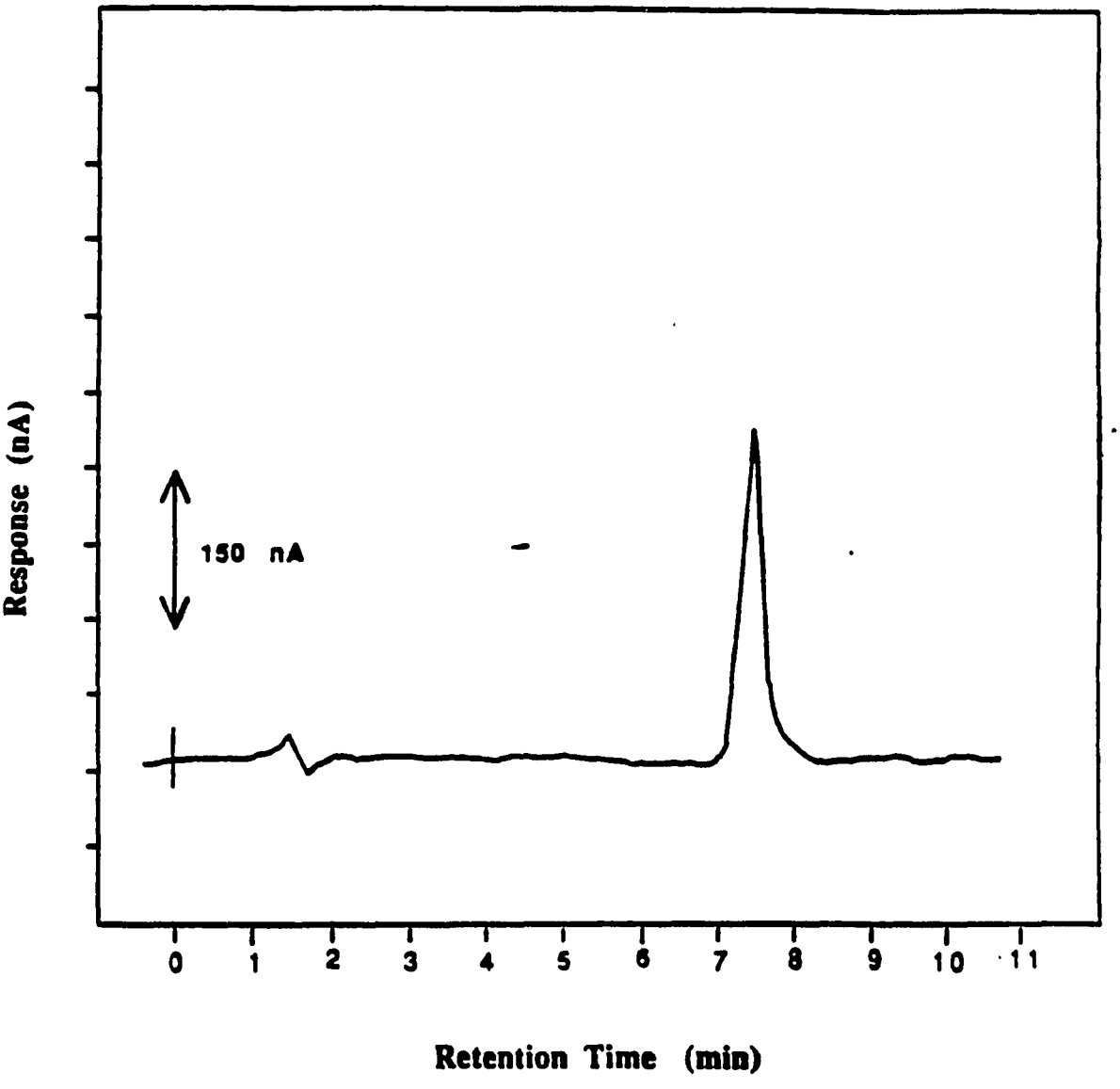

Figure 5. HPLC-ECD chromatogram of supercritical fluid extract of sawdust spiked with 44 µg anthraquinone. Chromatographic conditions as in Figure 1. Extraction conditions: SCFE at 8000 psi CO_2, 65 ^{0}C, 20 min.

sawdust sample, as shown in Fig. 6. Anthraquinone, peak 1, is clearly identifiable by its mass spectrum, shown in Fig. 7, along with the Wiley-NBS MS library (supplied with the Hewlett-Packard 1000 data system) spectrum of the compound (Fig. 8). With a column of poorer resolving power one would have difficulty separating anthraquinone from co-extractants, and in addition one would have to wait for all the peaks to emerge before injecting another sample. By comparison, the HPLC-ECD method is highly selective, sensitive, and rapid.

CONCLUSION

Anthraquinone is extracted quantitatively (extraction efficiency >90%) from Kraft paper and pine plywood sawdust using supercritical fluid at 8000 psi and 65 ^{0}C. Quantitative extraction requires only 20 min, does not lead to formation of emulsions or other phase separation problems as does solvent extraction, and is far faster than Soxhlet extraction, which requires at least 4 h to extract 82% of the anthraquinone. The anthraquinone is determined using reversed-phase HPLC with reductive electrochemical detection at a silver electrode at -1.1 V vs calomel. The minimum detectable quantity of anthraquinone at the silver electrode is 210 pg and response is linear over at least 4 orders of magnitude.

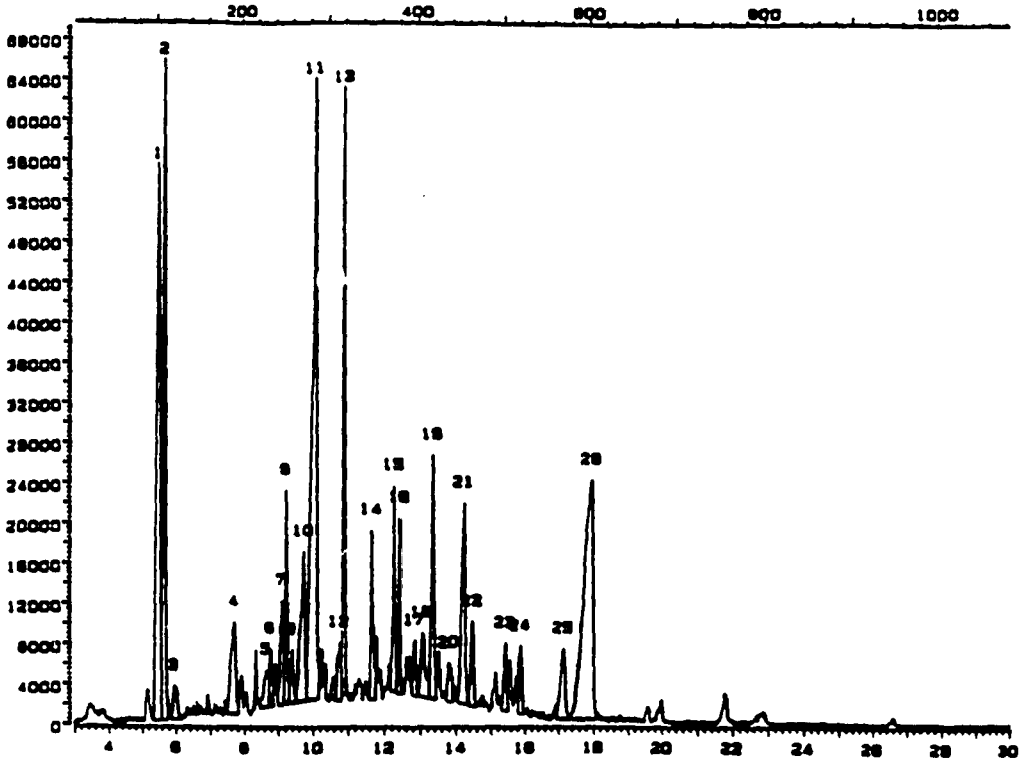

Figure 6. GC-MS chromatogram of supercritical-fluid extract of sawdust spiked with 0.44 μg of anthraquinone. Extraction conditions: 8000 psi CO_2, 65^{0}C, 20 min. GC conditions: 30 m x 0.025 mm I.D. DB-5 fused-silica capillary column; temperature, 150 ^{0}C for 2 min, then temperature programmed at 10^{0}C /min to 250^{0}C; carrier gas, He, 1 mL/min.

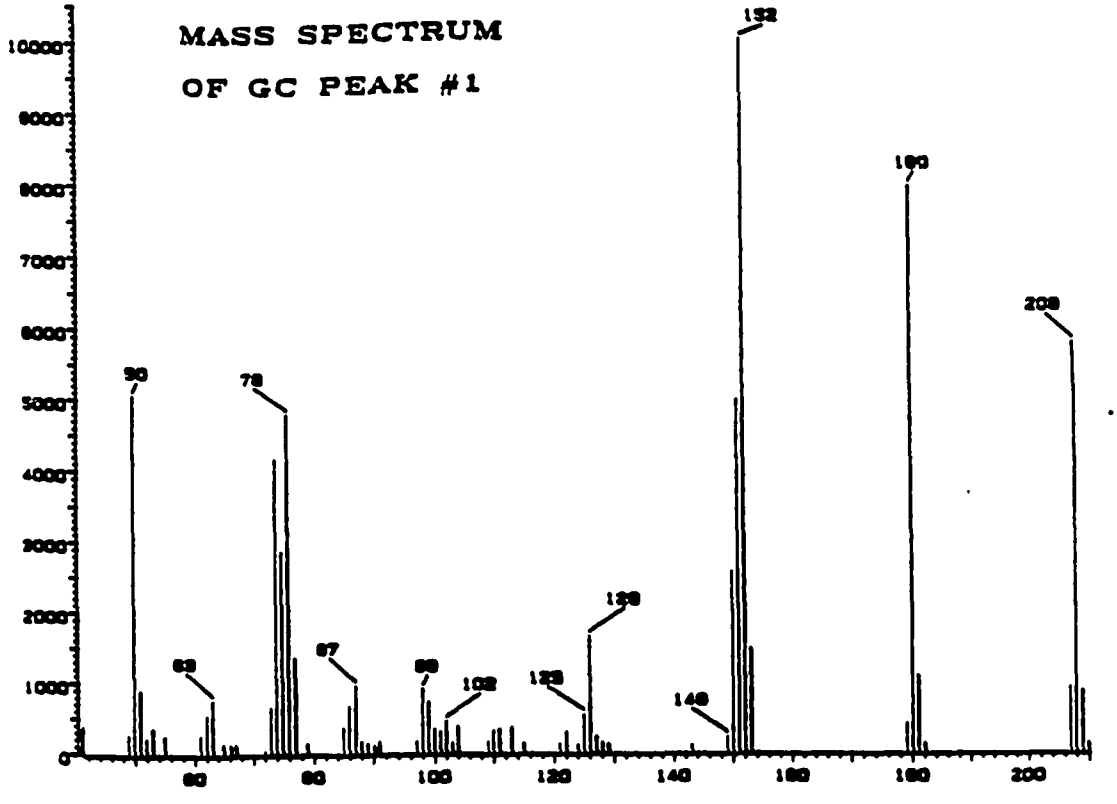

Figure 7. Mass spectrum of GC peak 1 in Figure 6. SCFE conditions : 8000 psi CO_2 , 65 ^{0}C, 20 min. GC conditions: 30 m x 0.025 mm I.D. DB-5 fused-silica capillary column; temperature, 150^0 C for 2 min, temperature programmed at 10 ^{0}C /min to 250^0 C; carrier gas, He, 1mL/min.

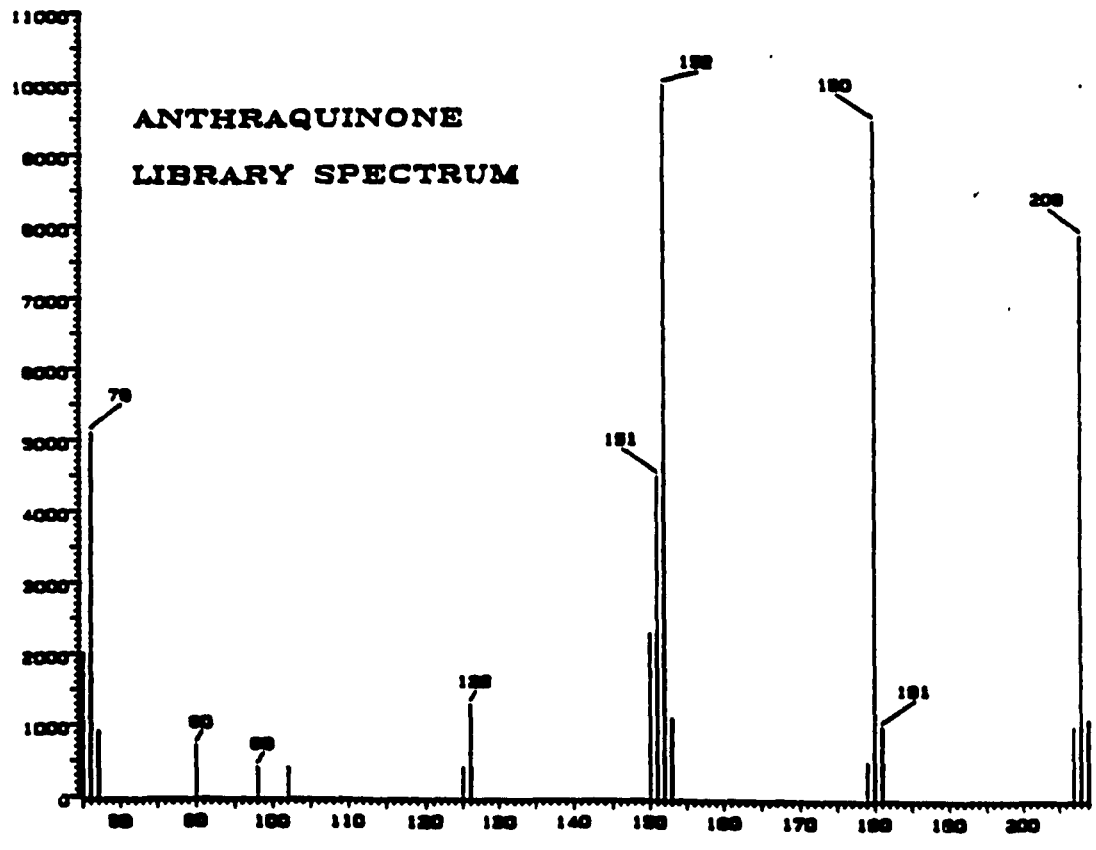

Figure 8. Library standard mass spectrum of anthraquinone.

Chapter 4

DETERMINATION OF MENADIONE IN ANIMAL FEED

INTRODUCTION

In long-term feeding studies of chemical substances, compound administration to laboratory animals is accomplished by incorporating the substance into the animals' feed. Analytical methods are required to monitor dose level, to verify uniform distribution in the feed mix, and to test for stability. Laboratory animal feeds are complex mixtures of diverse substances; if co-extracted, these excipients may interfere in the determination of the analyte of interest. Extensive sample clean-up procedures may then be required. The extraction of a lipophilic compound, menadione (vitamin K_3), from laboratory rat chow spiked with the compound, using supercritical fluid CO_2 is reported here. The extracted menadione is quantitated using reversed phase HPLC with an electrochemical detector (ECD). No clean-up of the extract was required.

Menadione (2-methyl-1,4-naphthoquinone) is a synthetic provitamin (1) which is converted into the active vitamin K_2 (menaquinone) in the liver, where it acts as a cofactor in the production of blood clotting factors (2). To combat a vitamin K deficiency in animals brought about by antibiotic or anticoagulant treatments, menadione can be administered by fortification of the feed (2). Present methods for the isolation of lipophilic vitamins from feeds and related materials involve time-consuming extraction steps, which introduces analytical uncertainty and limited ability to automate such assays (3). For example, the AOAC method (4) for tocopherol in foods and feeds requires an 8 hour Soxhlet extraction in ethanol, alkaline hydrolysis of the co-extracted lipids, and a multistage liquid-liquid partition procedure. Bourgeois, et al. (3) eliminated the solvent extraction steps by initially saponifying the lipids, and then isolating retinol and α-tocopherol on a commercial disposable cartridge packed with diatomaceous earth. We believe the need that exists for a simpler and cleaner isolation procedure for the fat-soluble vitamins in complex matrices is met by SCFE. A variety of wet chemical and instrumental procedures are available for the determination of menadione isolated from various media. Gas chromatographic (GC) methods, reviewed some time ago by Sheppard & Hubbard (5), are not highly reproducible and have been superseded by HPLC methods (6). While most HPLC procedures use a UV detector (UVD), greater specificity and sensitivity for K vitamins have been achieved with the ECD. The ease of the 2-electron reduction of menadione and related compounds has been known for some time (7), allowing use in HPLC flow cells of solid electrodes such as glassy carbon

(8), porous graphite (9), gold amalgam (10), and silver (10). HPLC-ECD has been applied to the determination of K vitamins isolated from rat liver tissue (9), human serum (8), and rat plasma (11). A dual ECD in the reduction/oxidation mode using coulometric/coulometric or coulometric/amperometric response, or coulometric/fluorimetric detection, offers as expected the highest sensitivity and selectivity towards plasma samples (12). The detection limits of these three modes of ECD are reported to be 150 pg, 280 pg, and 25 pg, respectively (12).

EXPERIMENTAL

Materials and solutions

Menadione was obtained from Sigma Chemical and was used without further purification. Acetonitrile (ACN), methylene chloride, and acetone were of HPLC grade. Water was glass distilled. The HPLC eluent was ACN/0.025 M aq. $NaClO_4$ (90/10, v/v). For sample spiking, a stock solution of menadione was prepared by dissolving 30.0 mg in 100 mL of CH_2Cl_2; this solution was then diluted with CH_2Cl_2 to make solutions in the 1.5 μg to 300 μg/mL range for HPLC standards. Solutions of menadione were protected from sunlight during storage and use. Crushed laboratory rat chow (Ralston-Purina Co., Type 5010-C) samples (500 mg) were fortified in the range of 200 ppm to 0.3% by adding 1 mL aliquots of CH_2Cl_2 solutions of menadione, mixing, and allowing the solvent to evaporate. Carbon dioxide was Linde bone-dry grade.

Instrumentation

The high pressure manifold used for SCFE work (Chapter 2), and a Varian HPLC with a silver working electrode have been already described (Appendix 1). To confirm the identity of menadione isolated by SCFE and that of HPLC peak, a Hewlett-Packard 5988 A GC/quadrupole mass spectrometer (GC/MS) system with a Hewlett-Packard 1000 data system was used with a 30-m x 0.25-mm i.d., DB-5 cross-linked methyl silicone bonded phase fused silica capillary column (J &W Scientific). The initial temperature, 150 ^{0}C, was held for 2 min followed by temperature programming at 10 ^{0}C/min to 250 ^{0}C. The carrier gas was He at 1 mL/min, and splitless injection was used.

RESULTS AND DISCUSSION

Hydrodynamic voltammogram

Figure (1) shows the hydrodynamic voltammogram on a silver electrode for repetitive injections of 20 µL of a 0.33 µg/mL menadione standard. The limiting current plateau was reached at approximately -0.75 V vs calomel, which was the potential used in all subsequent experiments. The 2-electron reduction is evidently quasi-reversible, because the value of $E_{3/4}$-$E_{1/4}$ is approximately 100 mV, intermediate between the values corresponding to reversibility (28.2 mV) and irreversibility (113 mV) (13).

Effect of flow rate

The variation of response with eluent flow rate is shown in Figure 2. For flows up to 1.67 mL/min, response increased with approximately one-third the power of the flow rate. According to Weber (14), this suggests the reduction current is limited by convective transport of menadione to the electrode. Because response becomes essentially independent of flow rate at flows higher than 1.7 mL/min, 2 mL/min was used in subsequent experiments.

Supporting electrolyte concentration

The ECD generally requires the presence of electrolyte to reduce solution resistance, but electrolyte suppresses solute solubility in the mobile phase and increases retention. As shown in Figure 3, detector response increased sharply with the concentration of $NaClO_4$ and peaked at about 0.025 M. At higher concentrations, the peak current waned, which might be attributed to increased peak broadening resulting from increased retention time and to a decrease in the diffusion coefficient attending the increase in $NaClO_4$ concentration.

Response of ECD to menadione

The ECD response parameters were determined by injecting 20 µL samples of standard solutions of menadione in ACN over the concentration range 0.03 µg/mL to 300 µg/mL. A log-log plot of peak current vs. weight of menadione injected was a straight line of unit slope over the range from 600 pg to at least 6 µg. The noise level was 0.05 nA, so that, at a signal-to-noise ratio of 3, the minimum detectable quantity (MDQ) was 125 pg. The ECD is thus about four times more sensitive than the UVD, which has a reported detection limit of 0.5 ng (9). It should be noted that this MDQ refers to the ECD, not the overall method.

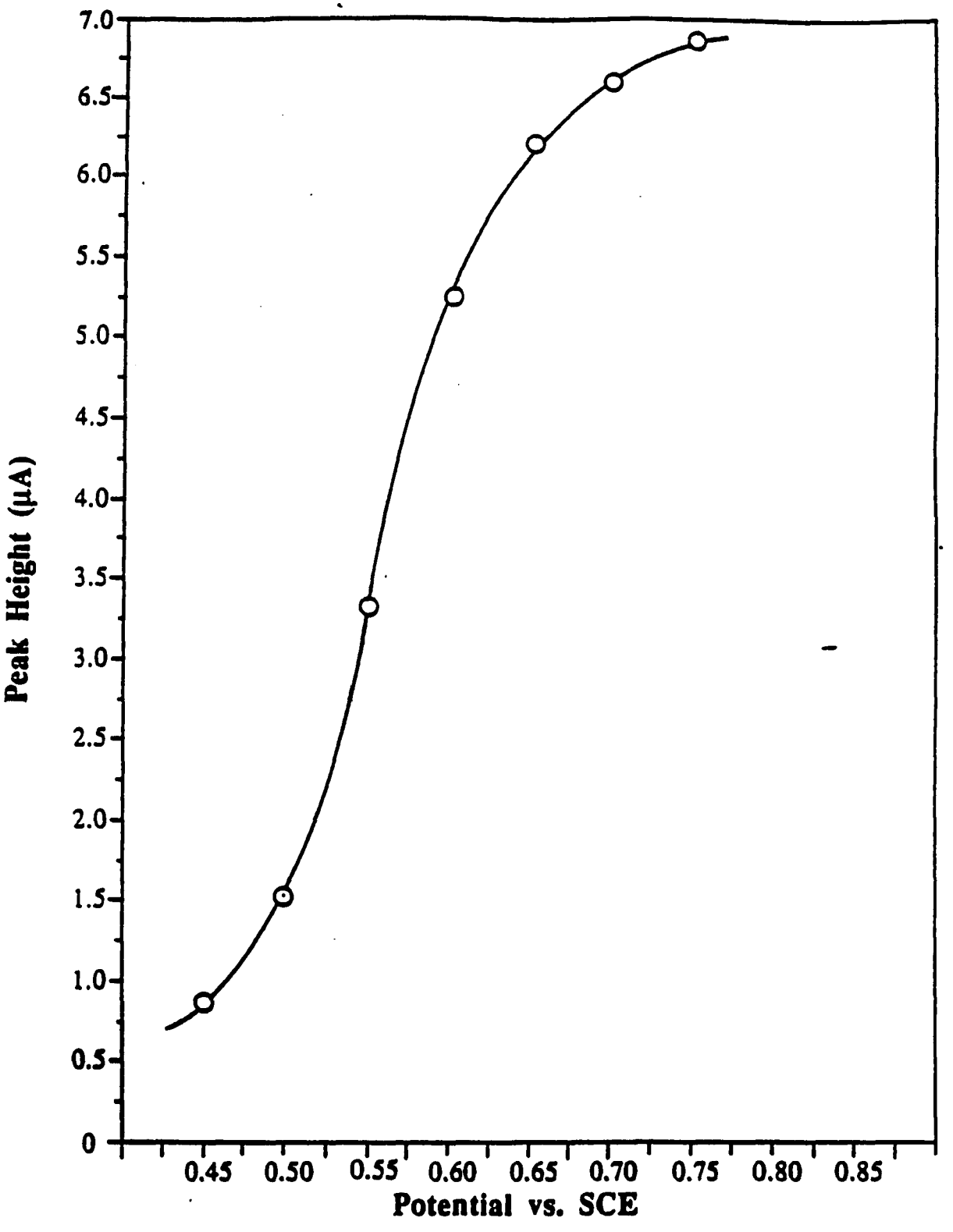

Figure 1. Hydrodynamic voltammogram of menadione on silver cathode. Column, 150 x 3.9 mm I.D. µBondapak C-18 (10 µm); Mobile phase, acetonitrile-aq 0.025 M $NaClO_4$ (90+10, v/v); Flow rate, 2 mL/min; sample size, 6.7 µg menadione per 20 µL MeCN injected at each potential setting.

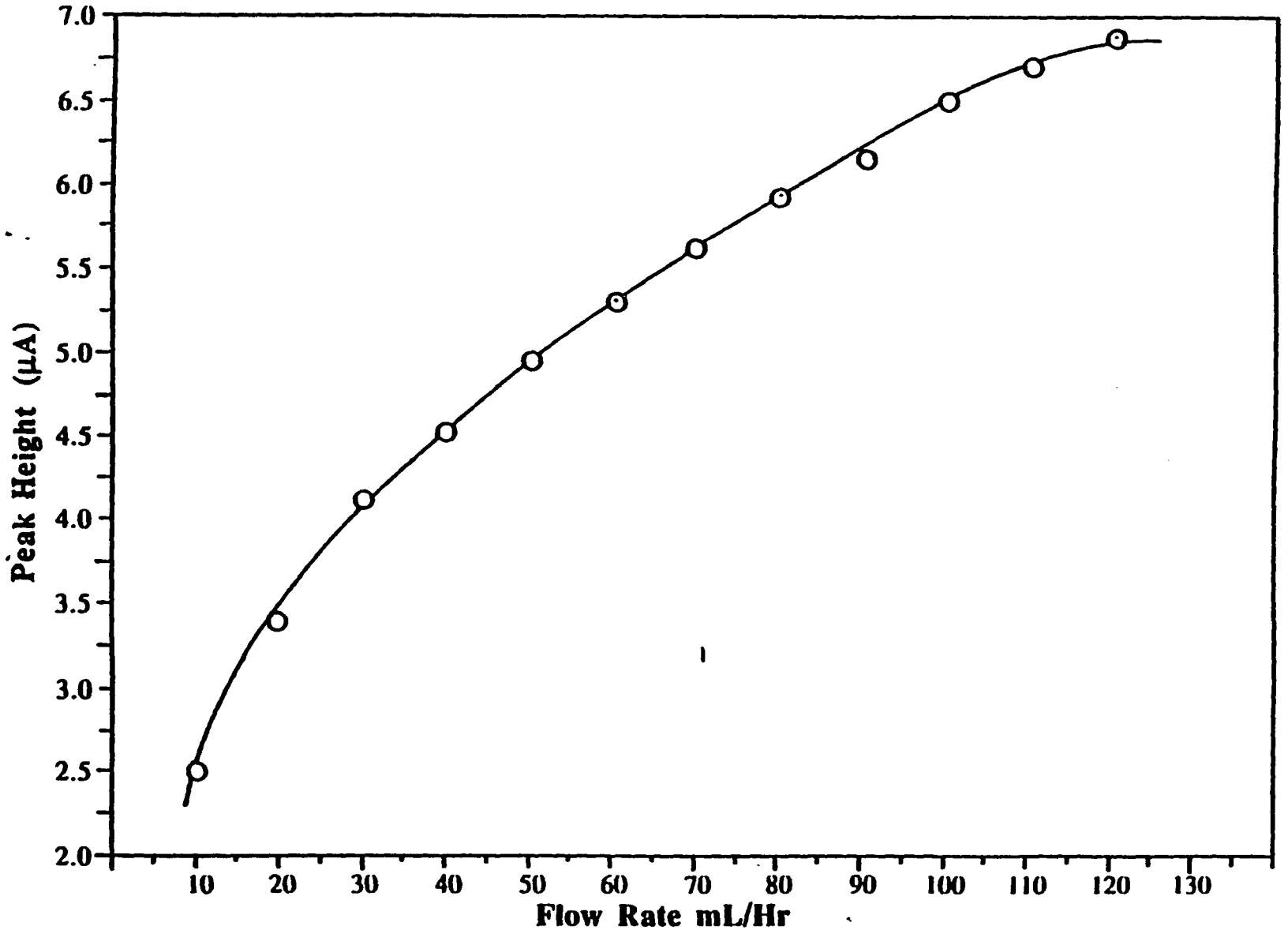

Figure 2. Variation of peak current with eluent flow rate. Chromatographic conditions: column, 150 x 39 mm I.D. µBondapak C-18; mobile phase, acetonitrile-aq 0.025 M $NaClO_4$ (90+10, v/v); Ag electrode potential, - 0.75 V *vs* SCE; Sample size, 6.7 µg menadione per 20 µL acetonitrile injected.

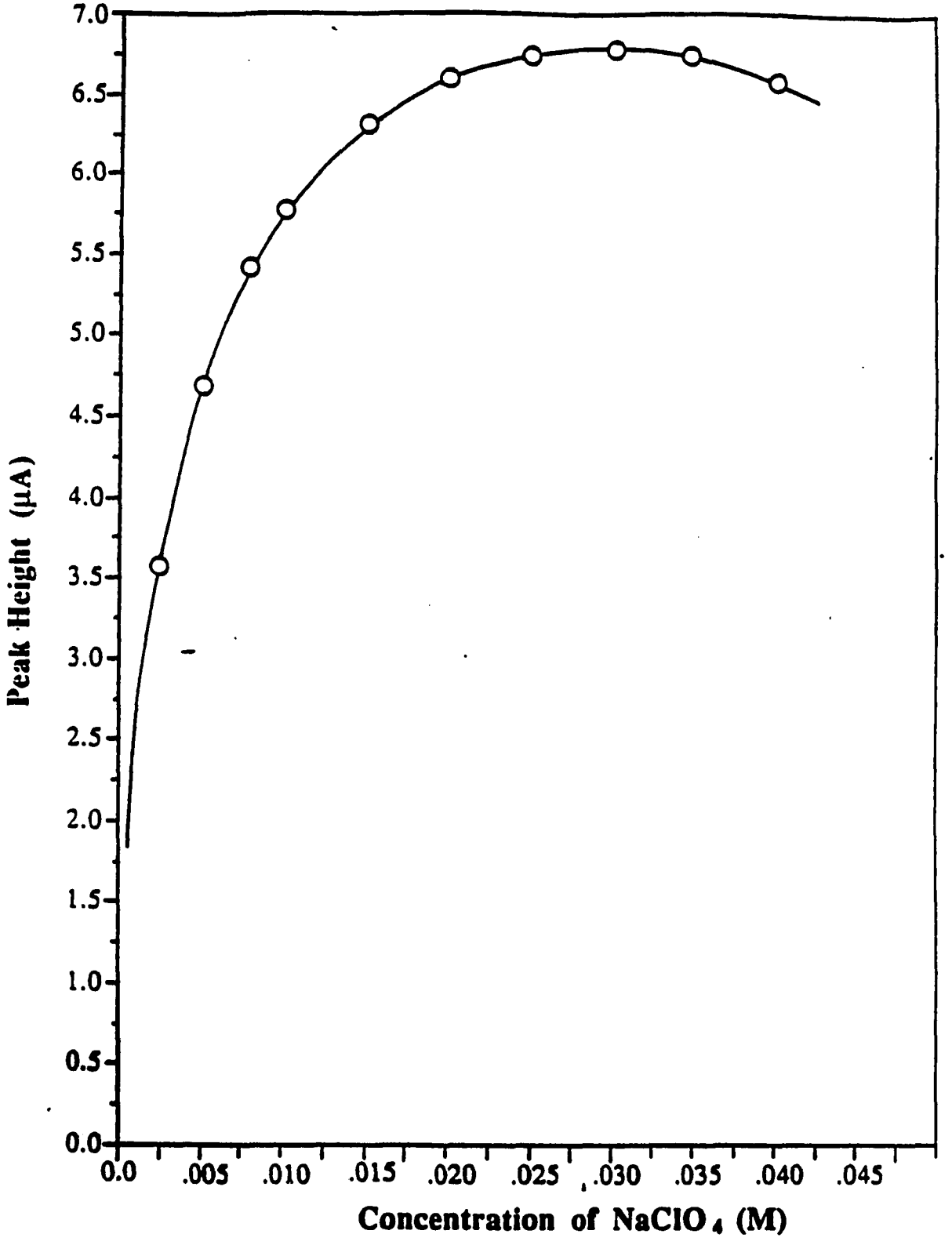

Figure 3. Effect of aqueous $NaClO_4$ electrolyte concentration on response. Chromatographic conditions: column, 150 x 3.9 mm I.D. μ Bondapak C-18 (10 μm); stock mobile phase composition, acetonitrile-water (90+10, v/v); flow rate, 2.0 mL/min; silver electrode potential, - 0.75 V vs SCE; sample size, 6.7 μg menadione per 20 μL acetonitrile injected at each mobile phase composition.

SCFE

In preliminary experiments the extraction of 1,4-naphthoquinone, a compound with SCFE behaviour identical to that of menadione (2-methyl-1,4-naphthaquinone) from filter paper was studied. As indicated in Tables I & II, 90% extraction of quantities added to a 2 cm. filter paper circle was achieved by extraction for 20 min at 8000 psi and 60 ^{0}C. The same conditions were used for the SCFE of the rat chow spiked with menadione at various levels. The results are given in table III. There was a certain low unextractable level of menadione that remained adsorbed on some component of feed matrix. This caused the relative recovery to suffer at levels below 0.2 mg/g of feed. To determine this unextractable level, six 0.5-g feed samples were spiked with 0.5 to 1.0 mg menadione in 0.1 mg increments. Each was extracted using the SCFE procedure. The plot of peak currents of the resulting HPLC-ECD peaks vs. milligrams of menadione added was a straight line. Extrapolation to zero peak current gave the nonextractable quantity, 10 μg menadione (i.e., 20 μg/g of feed). For actual samples containing menadione at levels below 0.2 mg/g , the 20 μg/g could be applied as a blank correction.The problem of unextractable menadione colud be resolved by adding polar entrainers such as dichloromethane directly to the animal feed.

Analytical Precision

To evaluate the precision of the method, menadione was added to triplicate 0.5-g feed samples at the 0.02 mg to 1.5 mg range, extracted, and determined by HPLC-ECD. As shown in Table III, the coefficient of variation ranged from 0.4 % to 4.6%, generally increased as the level of menadione decreased. The day-to-day precision was assessed by repetitive extractions of a feed sample spiked with 1.0 mg menadione/g of feed each day for five days. The average recovery was 90.5% with a coefficient of variation 2.2%.

Confirmation of HPLC peak identity

Figure 4 shows a typical HPLC-ECD chromatogram of an SCF extract of a rat chow sample spiked at 1 mg/g level. Only one peak was observed, which allowed the use of a strong mobile phase to shorten the retention time. Confirmation of peak identity was accomplished in three ways. First, HPLC-ECD of an SCFE of an unspiked feed sample produced no peaks at all. Second, addition of an increment of menadione to an extract produced a proportional increase in peak height with no increase in peak width. Third, the HPLC peak was collected and analyzed by GC-MS. Because the concentration in the collected peak was low, the use of selected ion monitoring MS mode (SIMS) was required. The SIMS chromatograms at six characteristic ions showed peaks at the same retention time as that of a menadione standard with the same relative peak heights

Table 1. Effect of SCFE Pressure on Extraction of 1,4-Naphthoquinone+ from filter paper

CO_2 Pressure (psi)*	Average Recovery (%)	RSD (n=3)
5000	51	3.1
6000	60	2.5
7000	85	2.3
8000	91	2.2

+ level of fortification = 100 μg

* 20 min equilibration, 60 °C

Table II. Effect of SCFE* Equilibration Time on Extraction of 1,4-Naphthoquinone+ from filter paper

Equilibration time (min)	Recovery (%)	RSD (n=3)
5	49	2.6
10	71	2.8
15	88	2.1
20	90	2.5

+ level of fortification = 100 μg

* 8000 psi CO_2, 60 °C

Table III. SCFE of Menadione from Rat Chow*

Menadione added (mg)	Average recovery (%)	Relative standard deviation
1.5	95	0.42
1.0	94	0.49
0.5	92	0.44
0.1	79	2.2
0.05	65	3.5
0.02	42	4.7

* Triplicate 0.5-g samples of rat chow spiked with menadione, extracted at 8000 psi CO_2, 60 ^{0}C, 20 min equilibration time.

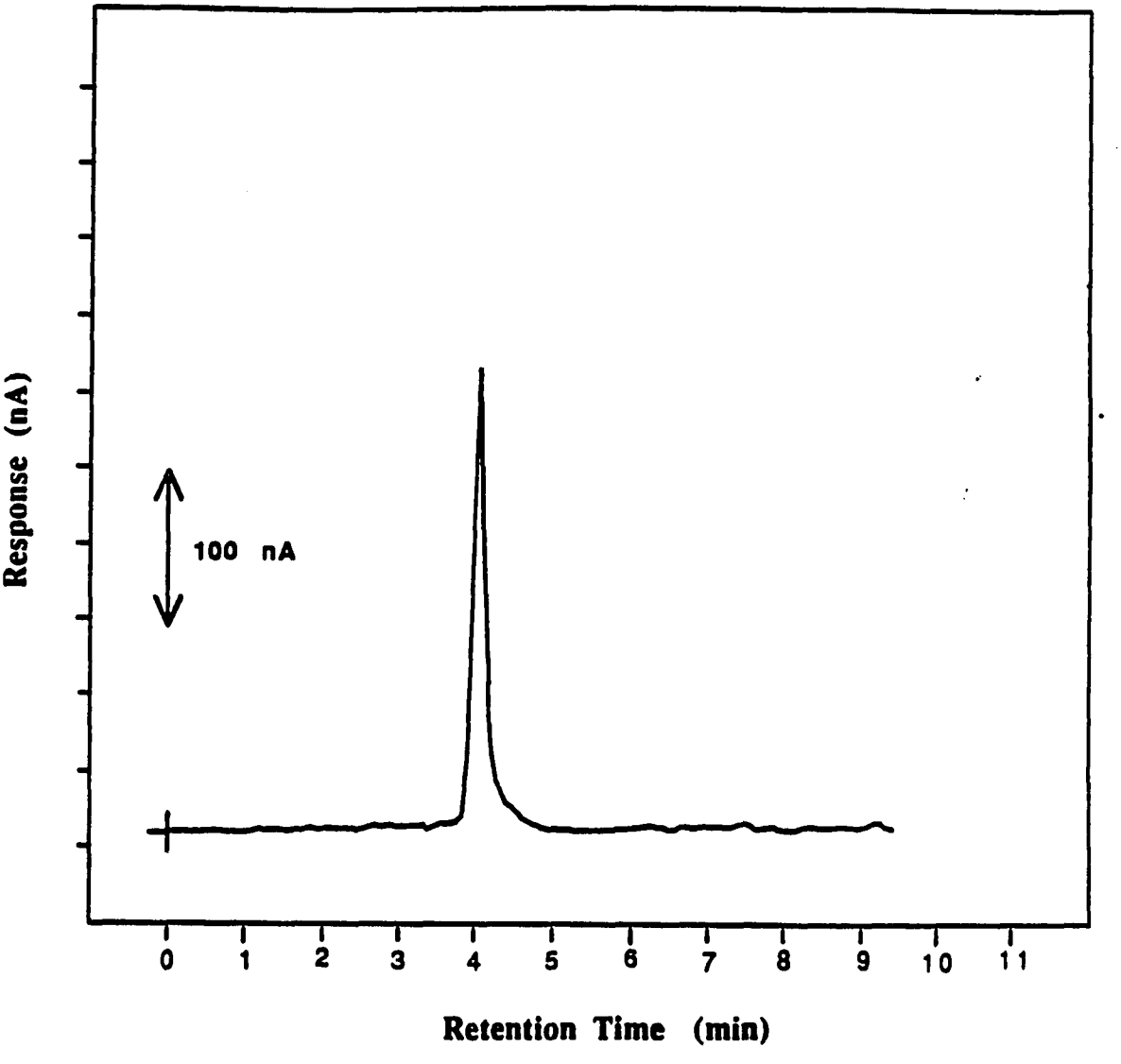

Figure 4. HPLC-ECD chromatogram of a supercritical-fluid extract of menadione fortified rat chow. Chromatographic conditions: column, 150 x 3.9 mm I.D. μBondapak C-18 (10 μm); mobile phase, 90/10 (v/v) acetonitrile -aqueous 0.025 M $NaClO_4$; flow rate, 2.0 mL/min; silver electrode potential, -0.75 V *vs.* SCE; sample size, 500 mg of rat chow spiked with 1 μg menadione/g of feed. SCFE at 8000 psi CO_2, 60°C, 20 min.

as the corresponding ions in the mass spectrum. Figure 5 shows a total ion GC/MS chromatogram of an SCF extract of rat chow spiked with 1.5 mg menadione/g of feed. The simplicity of the chromatogram is somewhat surprising and probably indicates the cleanness of the SCF extraction. The identity of peak 1 was confirmed by comparison of its mass spectrum with that of a standard. In figure 6, A is the mass spectrum of peak 1, B is the National Bureau of Standards-Wiley condensed mass spectral library mass spectrum of the compound.

CONCLUSION

Menadione (vitamin K_3) is extracted from spiked rat chow using supercritical fluid carbon dioxide at 8000 psi and 60 ^{0}C. Quantitative extraction requires only 20 min. The extraction does not suffer from the problems associated with conventional solvent extraction of lipophilic materials from animal feeds. Menadione is determined in the extract, which does not require any further clean-up, using reversed-phase high-performance chromatography (HPLC) with reductive mode electro- chemical detection at a silver electrode at -0.75V vs. calomel. The minimum detectable quantity by the detector is 125 pg of menadione, and the response is linear over at least 4 orders of magnitude; however the minimum quantity extractable is about 20 µg/g of feed. Repetitive extracts of a spiked feed sample over a five day period show an average recovery of 90.5% with a relative standard deviation of 2.2% at the 1 mg/g level.

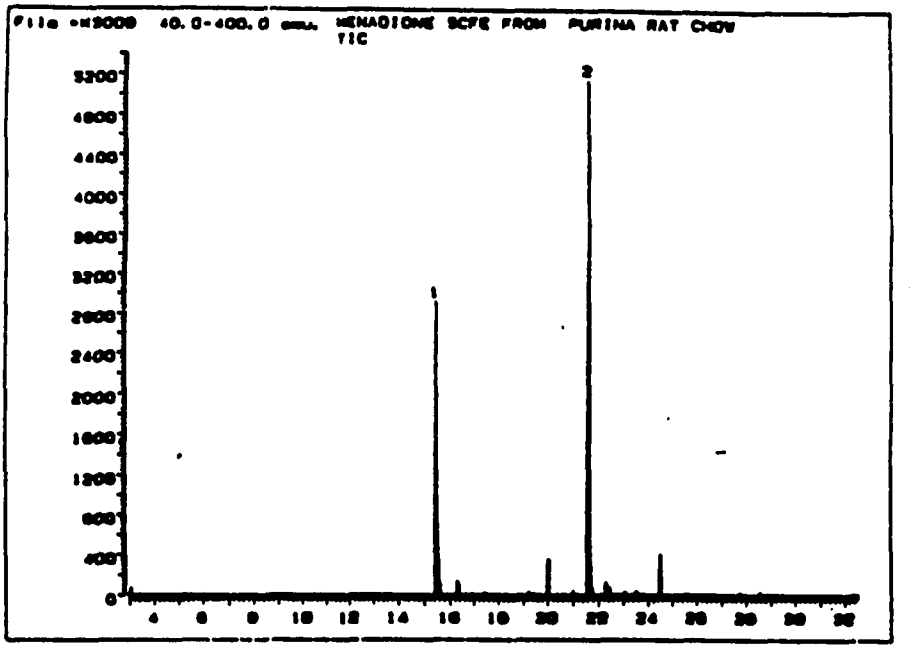

Figure 5. GC/MS total ion chromatogram of supercritical-fluid extract of menadione spiked rat chow. Peak identities as follows 1, menadione; 2, dibutylphthalate. 500 mg of rat chow spiked with 1.5 mg menadione/g of feed. SFE at 8000 psi, 60 ^{0}C, 20 min. GC conditions: 30 m x 0.025 mm I.D. DB-5 fused-silica capillary column; temperature, 150 ^{0} C for 2 min, then temperature programmed at 10 ^{0} C/min to 250 ^{0} C; Carrier gas, He, 1 mL/min.

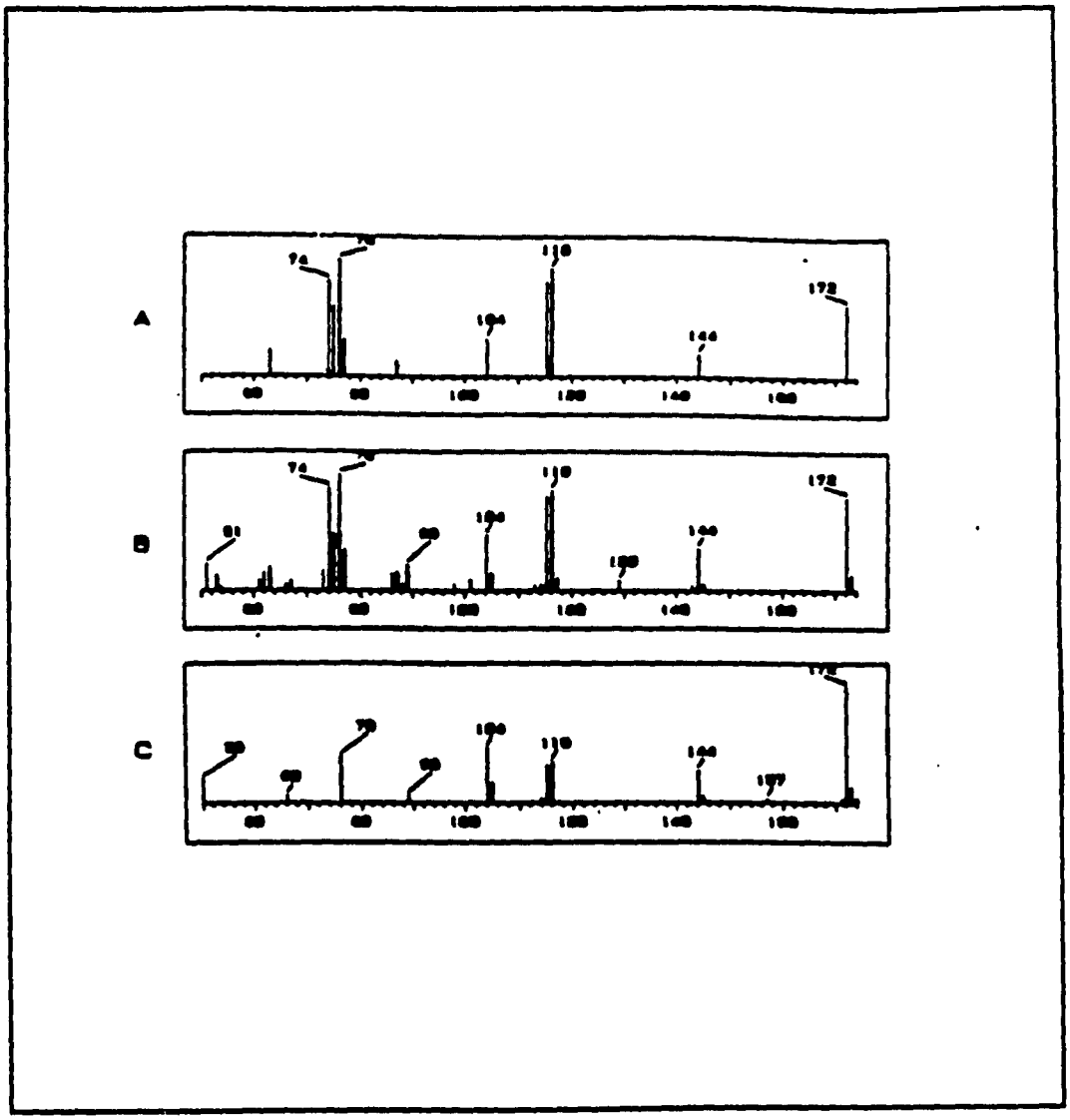

Figure 6. Comparison of mass spectra of recovered menadione with that of the library standard spectra. A = MS of supercritical-fluid extract of rat chow spiked with 1.5 mg menadione/g feed; B = MS of standard sample of menadione C= NBS-Wiley condensed mass spectral library mass spectrum of menadione.

Chapter 5

DETERMINATION OF VITAMIN K_1 IN THE POWDERED INFANT FORMULAS

INTRODUCTION

Despite the demands of the Infant Formula Act of 1980 (1), at present there is no standard method for determing vitamin K_1 (phylloquinone) in these food products. The original chick bioassay method (2) is not amenable to quality control-type procedures because it lacks precision and is expensive and time consuming.

Hwang (3) recently cited various instrumental methods for vitamin K_1 in infant formulas. Of these, HPLC with UV detection is generally superior in terms of speed, sensitivity to the ppb level, resolution of vitamin K_1 from other substances in the formulas, analytical precision, and procedural simplicity. Hwang (3) noted that reversed-phase liquid chromatography cannot resolve the cis-isomer of phylloquinone from the biologically active trans-isomer. Commercially available vitamin K_1 is about 90% trans-isomer. However, although normal-phase chromatography can resolve these isomers in standards and in liquid infant formulas, matrix interferences prevent determination of cis-isomer in powdered formulas (3).

An LC system with an electrochemical detector has been used for the determination of various K vitamins in rat liver tissue (4), human serum (5), and rat plasma (6, 7), but has not been applied to infant formulas. Published methods for the isolation of vitamin K_1 from the infant formula matrix usually describe a time-consuming initial alkali or enzyme hydrolysis of interfering lipids before extraction of the phylloquinone (8, 9). Hwang (3) simplified this procedure by first treating formula samples with ammonia and methanol, extracting with a methylene chloride-isooctane solvent mixture, and cleaning up the vitamin K_1 extract on a silica gel column. The determination step was normal-phase liquid chromatography with UV detection.

A rapid, single step extraction procedure using supercritical fluid CO_2 to isolate vitamin K_1 from powdered infant formulas has been developed. Phylloquinone in the extract is determined using reverse-phase liquid chromatography with a highly sensitive and specific electrochemical detector in the reductive mode.

EXPERIMENTAL

Chemicals

Acetonitrile, methylene chloride, and acetone were Baker HPLC grade (J & H Berge, South Plainfield, NJ). $NaClO_4$, reagent grade, was obtained from GFS Chemicals, (Columbus, OH); vitamin K_1 was supplied by ICN Biomedicals (Costa Mesa, CA), and was used without further purification as it showed only one HPLC peak. In house distilled water was used throughout the experiment. CO_2 was Linde bone-dry grade obtained from Prest-O-Sales (Long Island City, NY).

Vitamin K_1 standard solutions

A stock solution of 1 μg vitamin K_1/mL was prepared in acetonitrile-methylene chloride (95+5, v/v). Working standard solutions covering a concentration range 8-80 ng vitamin K_1/20 μL were prepared by several dilutions of standard in mobile phase. Exposure of samples and vitamin K_1 standards to visible light was kept at a minimum.

HPLC

HPLC was based on a Varian 8500 syringe pump, a Rheodyne 7125 injector, and an electrochemical detector. The details of design of the electrochemical cell, potentiostat and other accessories along with procedural details are described in Appendix 1. A 10 μm μBondapak (Waters Associates, Milford, MA), C_{18} column was used for chromatographic separations. The mobile phase comprised of acetonitrile-methylene chloride-aq. 0.025 M $NaClO_4$ (90+5+5, v/v). A silver working electrode polarized at -1.1 V vs. SCE was used as the working electrode for the reduction of vitamin K_1.

Supercritical Fluid Extraction

Powdered formula (1.0 g) was held in the extraction chamber between glass wool plugs. The extraction chamber was connected to the high preesure extraction assembly as described Chapter 2. The oven temperature was maintained at 60 ^{0}C. Initially, CO_2 was allowed to flow through the sample for 2 min. at 50 mL/min to flush out air . The exit valve was closed and the system was pressurized to 8000 psi. The time of equilibration at this pressure was 15 min. The exit valve was cracked opened carefully to let the extract-laden CO_2 depressurize across the silica trap. On completion of depressurization, the silica trap was washed with 30 mL CH_2Cl_2-CH_3COCH_3 (1+1, v/v), the washings were evaporated to dryness, and the residue reconstituted in 1 mL mobile phase.

HPLC and Determination

A 20 µL aliquot was injected into the LC system with an eluant flow rate of 2.0 mL/min, at ambient temperature, with the Ag electrode potential set at -1.1 V vs SCE Vitamin K_1 was the only peak detected in the extract, at a retention time of 8 min. The run time was 9 min for standards and samples. For quantitation, the height of the vitamin K_1 peak in the samples was compared with the calibration curve. The mobile phase comprised of acetonitrile-methylene chloride-aq. 0.025 M $NaClO_4$ (90+5+5, v/v) was thoroughly degassed with He was optimized to yield a obtain sharp peak for the analyte in a reasonable chromatographic run time.

RESULTS AND DISCUSSION

Extraction

The extraction conditions used, 8000 psi for 15 min at 60 ^{0}C were established during SCFE studies on 1,4 naphthoquinone and on other quinones and polycyclic aromatic ketones (10). Lower pressures and shorter equilibration times than these gave recoveries less than 90% (chapter 8). The efficacy of these results for vitamin K_1 was confirmed in preliminary trials in which 1 mL of the 1 µg/mL standard was added to Chromosorb W (Alltech Associates, Deerfield, IL), the solvent evaporated, and the vitamin K_1 extracted by SCFE. Under the above extraction conditions, recovery was 92% with a multiple extraction precision (RSD) of 3.0% (N=7). The recovery of vitamin K_1 from powered infant formulas was assessed by spiking samples of a soy protein-based formula and a milk-based formula with vitamin K_1, at spiking levels from 0.25 to 2.0 µg/g, in 5 to 7 increments. Each of these was then extracted using the SCFE technique, and vitamin K_1 was determined by LC with electrochemical detection. Recovery was established by comparing the resulting average nA/ng of the spike with the slope of the calibration plot (1.208 nA/ng). For the soy protein-based product, recovery was 94.4% with an RSD of 6.5%; and for the milk-based formula, recovery was 95.6% with an RSD of 7.4% (N=7).

LC Determination

Selection of mobile phase and working potential

The eluant contains CH_2Cl_2 to increase the solubility of vitamin K_1, which is essentially insoluble in water. Chromatographic peak shape was greatly improved by addition of

5% (v/v) CH_2Cl_2 to the acetonitrile-aqueous electrolyte eluent. The Ag working electrode potential used, -1.1 V vs SCE, was established from the hydrodynamic voltammogram (HDV). The HDV is generated by setting the working electrode potential at values from -0.6 to -1.20 V at 0,05 V increments, and injecting a 20 µL aliquot of the vitamin K_1 standard solution at each potential. The plot of peak current vs applied potential is the HDV, which is reproduced in Figure 1. The limiting current plateau was reached at -1.10 V vs SCE. The detailed shape shows the 2-electron reduction to be quasi-reversible (11).

Effect of flow rate

The flow rate and supporting electrolyte concentration used (2 mL/min and 0.025M $NaClO_4$) were established in earlier work on menadione (10, chapter 4) . For flow rates less than 100 mL/h, response increased with the 1/3 power of the flow rate, indicating a convective mass transfer-limited current (12). Above this flow rate the response was independent of the flow rate. Similarly, increasing the supporting electrolyte reduced solute retention time and solute diffusion coefficient. Shorter retention times produced narrower peaks and thus greater peak height, whereas a slower diffusion coefficient reduced mass transfer rate. Peak height response vs electrolyte concentration passed through a maximum, in this case at a $NaClO_4$ concentration of 0.025 M.

A typical LC-electrochemical detector chromatogram is shown in Figure 2. The sample was 1 g of the milk protein-based product, and it is evident that vitamin K_1 is the only compound extracted which is detectable by the reductive mode electrochemical detector. The peak corresponds to about 45 ng vitamin K_1. The minimum detectable quantity of the vitamin, i.e., the weight that produces a signal 3 x the noise level, is 80 pg. The electrochemical detector is 4 x times more sensitive than the UV detector which is reported to be have a detection limit of 0.3 ng (3). The response of eletrochemical detector to vitamin K_1 is linear up to at least 8 µg, i.e., a linear dynamic rage of at least 10^5. The upper boundary is established by the solubility of vitamin K_1 in mobile phase; higher amounts could presumably be measured by increasing the amount of CH_2Cl_2 in the eluant.

For comparison, 10 g of the soy protein-based product spiked with 10 µg vitamin K_1/g of the sample was extracted using the SCFE procedure, and the extract was analyzed by reverse-phase liquid chromatography with a UV detector at 248 nm. A different (and

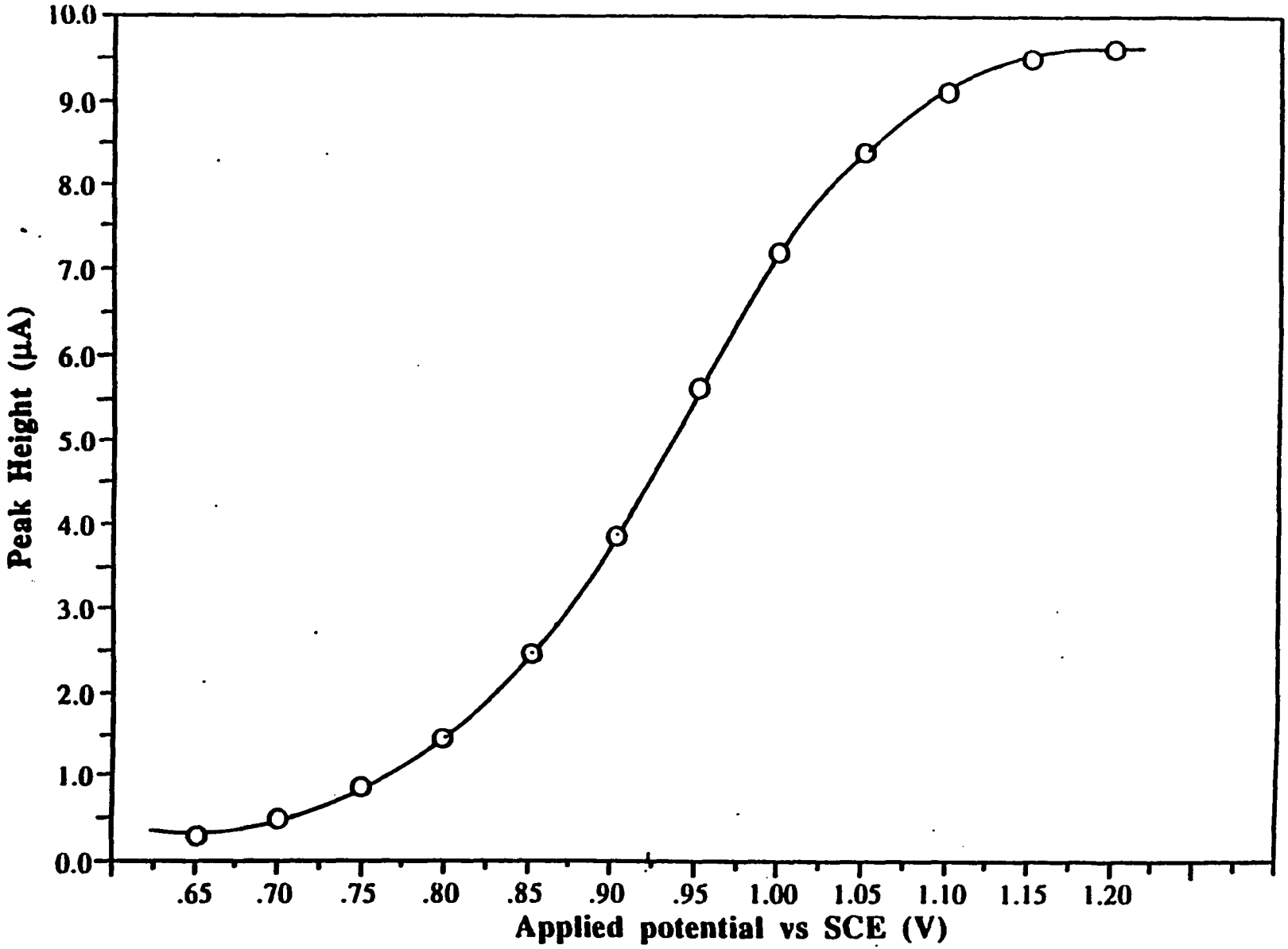

Figure 1. Hydrodynamic voltammogram of vitamin K-1 on silver electrode vs SCE. Column: 150 x 3.9 mm µBondapak C-18. Mobile phase: acetonitrile-methylene chloride - aq. 0.025M $NaClO_4$ (90+5+5, v/v). Flow rate: 2 mL/min. Sample size: 20 µg vitamin K-1 per 20 µl mobile phase injected at each potential setting.

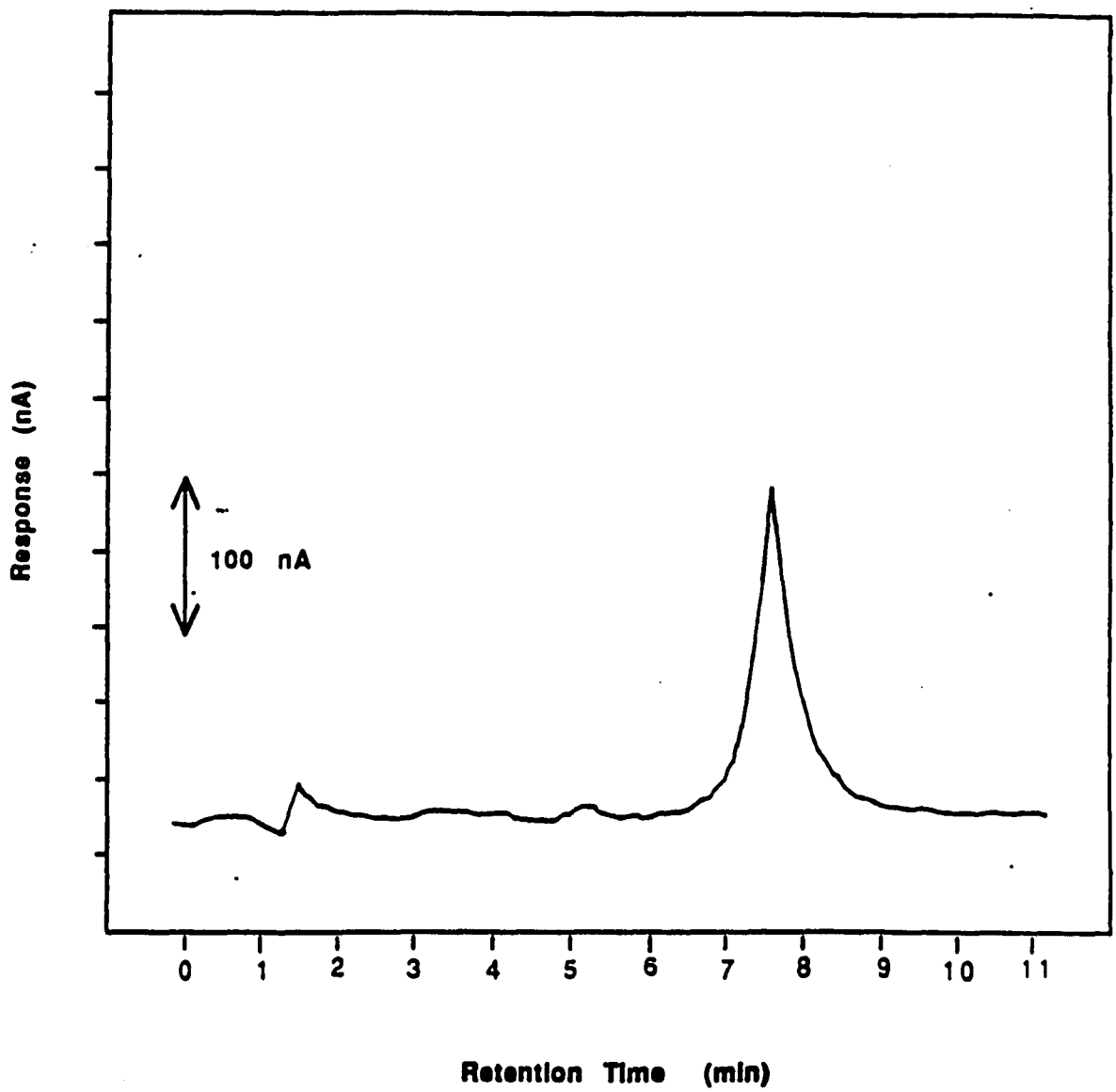

Figure 2. HPLC-ECD chromatogram of vitamin K-1 extracted from a commercially available milk-based fortified formula by SFE technique. Extraction conditions: SFE at 8000 psi CO_2, 60° C, 20 min. Chromatographic analysis conditions as in Figure 1.

newer) C-18 column was used with mobile phase lacking the $NaClO_4$ electrolyte. The chromatogram is shown in Figure 3. The peak corresponds to approximately 5 μg vitamin K_1. The supercritical fluid CO_2 is extracting more compounds than just the vitamin, but the presumably polar co-extracted materials are not electroactive under the conditions used.

Application to Powdered Infant Formulas

The method was successfully applied to a milk-based and a soy protein-based powdered infant formula (both purchased locally). Vitamin K_1 levels of 0.87 and 0.95 mg/g, respectively, were found (Table1). The package label states the level of vitamin K_1 in both products to be 15 mg/100 Cal, which is equivalent to about about 0.8 mg/g. An attempt was made to apply the method to liquid formulas, by using a larger extraction chamber (12 in x 3/4 in. id) packed with Chromosorb W to which 7.0 mL of a liquid product was added. No vitamin K_1 was detected in the elute from the silica trap. We believe the problem was caused by co-extraction of water, which saturates the trap and renders it ineffective for vitamin K_1. Perhaps, water could be selectively removed first by extracting the liquid formula at low pressures (i.e., 3000 psi) where the extraction of vitamin K_1 is essentially zero, then the sample could be extracted again at 8000 psi.

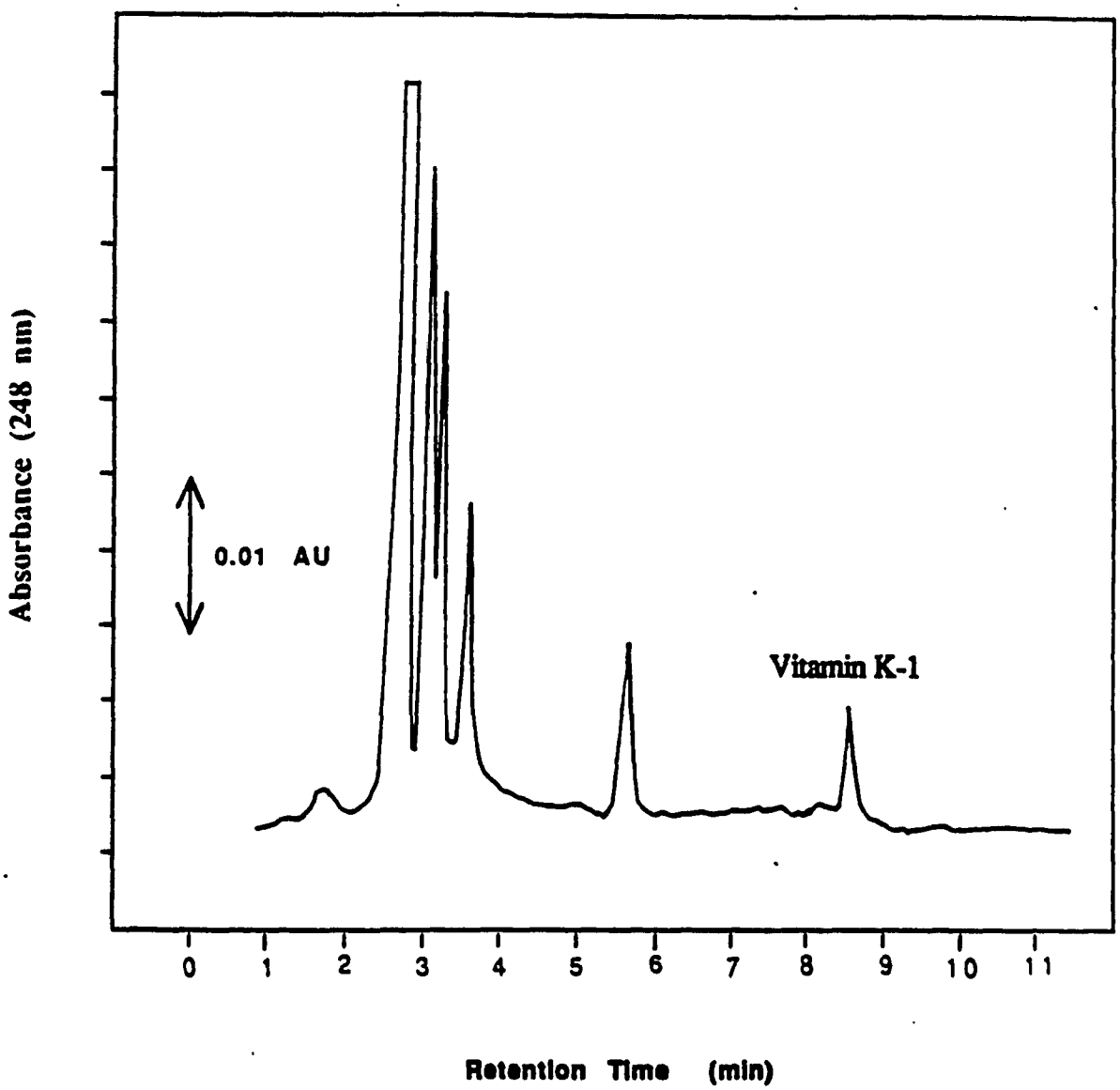

Figure 3. Liquid chromatogram with UV detection of added vitamin K-1 extracted from soy protein-based infant formula by SFE technique. Sample spiked with 10 µg vitamin K-1/g product. LC conditions: Perkin-Elmer 4000 LC system equipped with UV detector at 248 nm, 0.10 AUFS. Column, 15 cm x 4.6 mm I.D. Whatman C-18, with acetonitrile-methylene chloride-water (90+5+5, v/v) eluant. Flow rate 2 mL/min.

TABLE. 1
Results of LC Determination of Vitamin K_1 in Two infant Formulas

Product	K_1 found	% of declared	RSD (n=3)
Soy protein-based	0.95 mg/g	118	6.9
Milk-based	0.87 mg/g	108	8.2

CONCLUSION

Vitamin K_1 is extracted from commercial soy protein-based and milk-based powdered infant formulas by using supercritical fluid extraction with CO_2 at 8000 psi and 60 ^{0}C. Quantitative extraction required only 15 min, and did not suffer from from problems associated with conventional solvent extraction of lipophilic materials from media such as formulas. Vitamin K_1 was determined in the extracts by using reversed-phase LC with reductive mode electrochemical detection at a silver electrode polarized at -1.1 V vs SCE. LC run time was 9 min.The minimum detectable quantity was 80 pg, and response was linear over at least 5 orders of magnitude. Recovery of vitamin K_1 from a milk-based powdered formula was 95.6% with RSD of 7.4%, and from a soy protein-based product, 94.4% recovery with a RSD of 6.5%.

Chapter 6

Determination of retinyl palmitate (vitamin A) in ready-to-eat cereals

INTRODUCTION

The retinyl palmitate form of vitamin A is most commonly used for the fortification of commercially available ready-to-eat cereals typically at concentration levels of 25-125 µg/g (1). The isolation of retinyl palmitate from ready-to-eat cereals is complicated by several factors. Most important are the low fortification levels of retinyl palmitate and the presence of other extractable vitamins such as E, K_1, and D, in addition to the presence of lipids, antioxidants, and emulsifiers in the matrix. Further, retinyl palmitate is sensitive to heat, light and oxygen. Thus a method employed for the isolation of retinyl palmitate should be able to isolate selectively the analyte under gentle conditions without causing its thermal- or photo-degradation. Several methods for the isolation of this vitamin can be found in literature (1-4).

The most common sample preparation method (AOAC) (5) includes a multi step procedure, involving saponification, liquid-liquid extraction and further clean-up on an open silica column. The alkali hydrolysis for the removal of fat involves use of high temperature (80-90 ^{0}C) and above and high pH (>10) that may result in denaturing of vitamin A activity. The multiple steps involved in the procedure result in poor recoveries of analyte, and are prone to various analytical uncertainties. Moreover, the method is labor intensive, unselective and as such can not be automated.

In place of the high temperature alkaline hydrolysis, Barnett and co-workers investigated enzymatic hydrolysis for the simultaneous isolation of vitamins A, D_2, D_3, E, and K_1 in infant formula. The enzymatic hydrolysis is performed at room temperature and at neutral pH and yields a predictable and controllable product mix (6). However, the process is still time-consuming and requires further clean up before the extract can be analyzed.

Holasiva and Blattna reported fast fractionation of retinol, retinyl palmitate, and carotene from other fat-soluble vitamins in fortified margarine using a single Sephadex LH-20 column using high pressure gel permeation chromatography (7). They achieved quantitative recovery of the retinyl ester without saponification and thus avoided conditions which might lead to degradation of retinyl palmitate in the sample. Retinyl palmitate and ß-carotene have been selectively isolated from the bulk lipid in a margarine sample using a µStyragel column (8). Landen isolated retinyl palmitate from breakfast cereals using two or more µStyragel columns connected in series (9). Gel permeation chromatography was used to isolate retinyl acetate and ergocalciferol from the bulk of the lipid material in a

coconut oil sample (SRM 1563) and a cod-liver oil sample (SRM 1588) by Brown-Thomas et al. (10). In this method, vitamin fractionation was achieved on an semi-preparative amino/cyano column prior to fat elimination on a polystyrene/divinylbenzene gel column using a mobile phase composed of 30% methyl tert-butyl ether in methylene chloride. In spite of the advantage of being non-destructive, GPC still is a time consuming method. Thus a need exists for the development of a single step, rather straightforward, clean and fast sample preparation method for isolation of retinyl palmitate from breakfast foods.

For the quantitation of vitamin A, a number of colorimetric methods are still in use. An AOAC approved method involves formation of an unstable blue complex with vitamin A and corrosive antimony trichloride. The method is highly unselective as a number of sterols present in the matrix can form a blue complex with antimony trichloride. Since the complex formed is unstable the results have poor reproducibility (11). Similarly, the various other colorimetric and fluorimetric methods currently in use are not very selective; sensitive or precise (12, 13, 14).

Recently, HPLC methods involving high resolution columns have become very popular for the chromatographic separation and determination of various fat soluble vitamins such as A, K, E, and D_2. The use of HPLC for this purpose in all its forms, i.e., normal phase, reversed phase and nonaqueous reversed phase is documented in the literature (15-21). Thompson and Maxwell quantitatively determined vitamin A in various lipid-rich matrices using a reversed phase HPLC method on a 10 μm LiChrosorb column after saponification, followed by hexane extraction of the matrix (15). Egberg reported HPLC separation and quantitative determination of all cis and trans isomers of vitamin A on a Vydac 10 μm ODS column (16). Cohen and Lapointe determined vitamins A, K, D and E from various animal feeds on a μ-Bondapak C_{18} column (17). The reversed phase HPLC methods are time consuming since the run times are long, and they require a preliminary purification step for lipid removal before the extract can be analyzed. The droplets of fat and other viscous materials can easily be retained on the column surface resulting in shortened column life, occasional peak ghosting and high operating back-pressures.

Normal phase HPLC methods do not suffer from the problems of fat contamination of columns. Dennison and Kirk described a normal phase procedure on μ-Porasil after saponification and subsequent extraction of the sample with hexane for the analysis of vitamin A as retinol in cereal products. These authors reported that the presence of

carotenoids, various food additives, and vitamin A oxidation products did not interfere (18). Similarly Head and Gibbs determined vitamin A activity in saponified food composites by HPLC on a 10 μm LiChrosorb Si (60) column with gradient elution (19). Thompson et al. described an HPLC method in which the saponification step was replaced by a general fat extraction procedure . In this method, the ground sample was mixed with water and extracted with directly hexane. After centrifugation a 100 μL aliquot of the extract was removed for subsequent separation on a LiChrosorb Si (60) column using ethyl ether-hexane (2+98) as the mobile phase (20). Widicus and Kirk also reported on a normal phase method in which crude extracts from breakfast cereals are directly chromatographed on a μ-Porasil column, using an isocratic mobile phase of hexane-chloroform (85+15) with a flowrate of 1.5 ml/min (21, 22). Though straight-forward and relatively free from fat contamination problems, normal phase HPLC methods are not easily compatible with many sensitive and selective separation and detection schemes such as those involving electrochemical detection after HPLC separation.

To overcome these problems a number of non-aqueous reversed-phase (NARP) HPLC methods have been developed (23, 24, 25). In these methods, the vitamin A extracts obtained from GPC lipid fractionation were separated either on a Zorbax ODS or μ-Bondapak C-18 column using methylene chloride-acetonitrile-methanol (30+69+0.2, v/v) and quantitated using a UV detector. Of all the methods described above, none of them can claim to be a simple, precise and accurate.

To circumvent the problems associated with alkali/enzymatic hydrolysis followed by multi-step liquid-liquid extraction and then HPLC detection, we have developed a single stage, fast and straight-forward method with low lipid carry-over and minimal manual intervention. The method is the direct supercritical fluid extraction of retinyl palmitate from ready-to-eat breakfast food cereals under very mild conditions of temperature and in the absence of light. The extract, without any further clean-up, is fractionated using reversed phase HPLC on a C_8 (Altex) analytical column followed by detection by the highly sensitive and selective electrochemical oxidation at a glassy carbon electrode.

EXPERIMENTAL

Retinyl palmitate in oil was purchased from Sigma Chemical Co. (St. Louis, Mo). HPLC-grade acetonitrile and isopropanol were obtained from J. T. Baker (Phillipsburg, NJ). In-house glass distilled water was used throughout the study. Reagent grade $NaClO_4$ was obtained from Fisher Scientific (Pittsburgh, PA) and was used without further purification.

Preparation of standards

A stock solution of retinyl palmitate was prepared in isopropanol at a concentration of 350 µg/mL. Working solutions ranging from 3.5 to 35.0 µg/mL were prepared by diluting the stock solution with mobile phase. All solutions were kept in dark to avoid the photodecomposition of retinyl palmitate.

Apparatus

The laboratory-constructed HPLC used in the study consisted of a Varian 8500 syringe-pump, a Rheodyne 7125 injector, a 15 cm. x 4.6 mm id Altex C_8 analytical column and an amperometric thin-layer detector (Appendix1) equipped with a glassy carbon electrode. The working potential was set at + 1.2 V vs SCE. The electrochemical response was recorded on a Linear Model 1200 strip chart recorder. A peak detector (Appendix 1) was used to store individual peak heights for quantitation. The mobile phase was comprised of methanol-aetonitrile-aq. 0.05 M $NaClO_4$ (45/45/10, v/v). Prior to use the mobile phase was filtered and thoroughly deoxygenated with nitrogen according to procedure of Kissinger (26). The flow rate through the column was 2 mL/min and the column was maintained at ambient temperature.

Sample preparation procedure

Breakfast cereals were obtained from local retail stores. Prior to analysis, bulk composite samples were formed by mixing samples from two different lots. Typically, a two gram sample of each cereal was held in place in the extraction chamber between two glass wool plugs. The chamber was sealed and inserted into the high pressure extraction manifold (Chapter 2). The samples were extracted with carbon dioxide at 8000 psi and 60 ^{0}C for 15 min. Following depressurization, the extracted analytes along with silica gel were transferred to a syringe fitted with a filter and washed with 30 mL of isopropanol. The crude extract was evaporated in a rotary evaporator maintained at 45 ^{0}C, reconstituted in mobile phase and 20 µL aliquot of the resulting solution was injected onto the analytical

HPLC column. In order to evaluate the accuracy and precision of the method ground cereals such as corn, wheat or oat were fortified with vitamin A palmitate at concentrations ranging from 10-125 μg/g. The vitamin A was then isolated by SCFE procedure as described above and analyzed in triplicate using the carbon-based electrochemical detector.

RESULTS AND DISCUSSION

Chromatography

Vitamin A palmitate can be separated by normal-phase as well as by reversed-phase chromatography using a C_8 (Altex) analytical column. During preliminary studies, we investigated reversed-phase separations using a mobile phase composed of acetonitrile-water (90/10, v/v) containing 0.025 M sodium perchlorate. However, with this solvent system, poor peak shapes and long retention times were obtained for vitamin A palmitate. The addition of isopropanol to the mobile phase provided a markedly improved peak shape for vitamin A and compressed the separation of vitamin A from interferences into the smallest possible time span. The role of isopropanol, in an acetonitrile-water mixture (82/18, v/v), on vitamin A retention time is shown in Figure 1. An isopropanol content of 45% (v/v) in acetonitrile/water (45/10, v/v) offers the best results with respect to retention and selectivity. Under these conditions, the retention time for vitamin A is 10 min with a capacity factor (k') of 9.0. The effect of both isopropanol and acetonitrile together is dramatic; a change in isopropanol content of only 5% centered about the 45% level can change the retention time of vitamin A from 9.5 min to 20 min.

Electrochemical characteristics of vitamin A

Hydrodynamic voltammogram

A chromatographically-generated HDV was constructed by plotting applied potential vs. peak current obtained by repeated injections of 0.55 μg retinyl palmitate solution. A typical HDV for vitamin A at 2 mL/min is shown in Fig 2. The peak current increases as the potential is increased until a plateau is reached around +1.2 V vs SCE. Hence a voltage of +1.2 V vs SCE was used throughout this study.

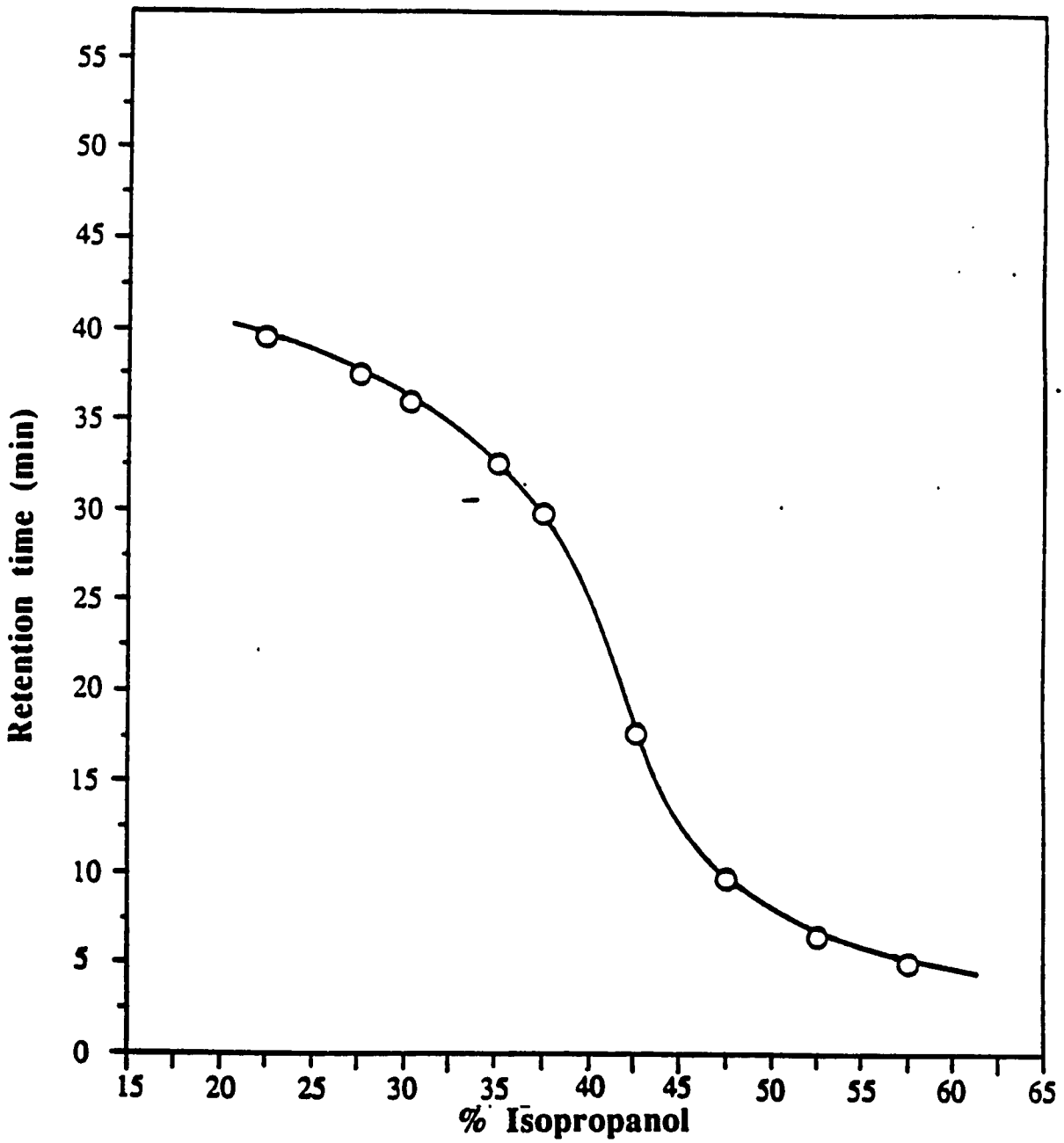

Figure 1. Dependence of vitamin A palmitate retention on isopropanol content of mobile phase. Chromatographic conditions: column, 150 x 4.6 mm I.D. Altex C-8 (10 μm); flow rate, 2.0 mL/min, silver detector electrode potential, 1.2 V *vs* SCE; mobile phase, each mobile phase prepared by mixing appropiate volumes of pure isopropanol and a stock aqueous-acetonitrile (18/82, v/v) solution.

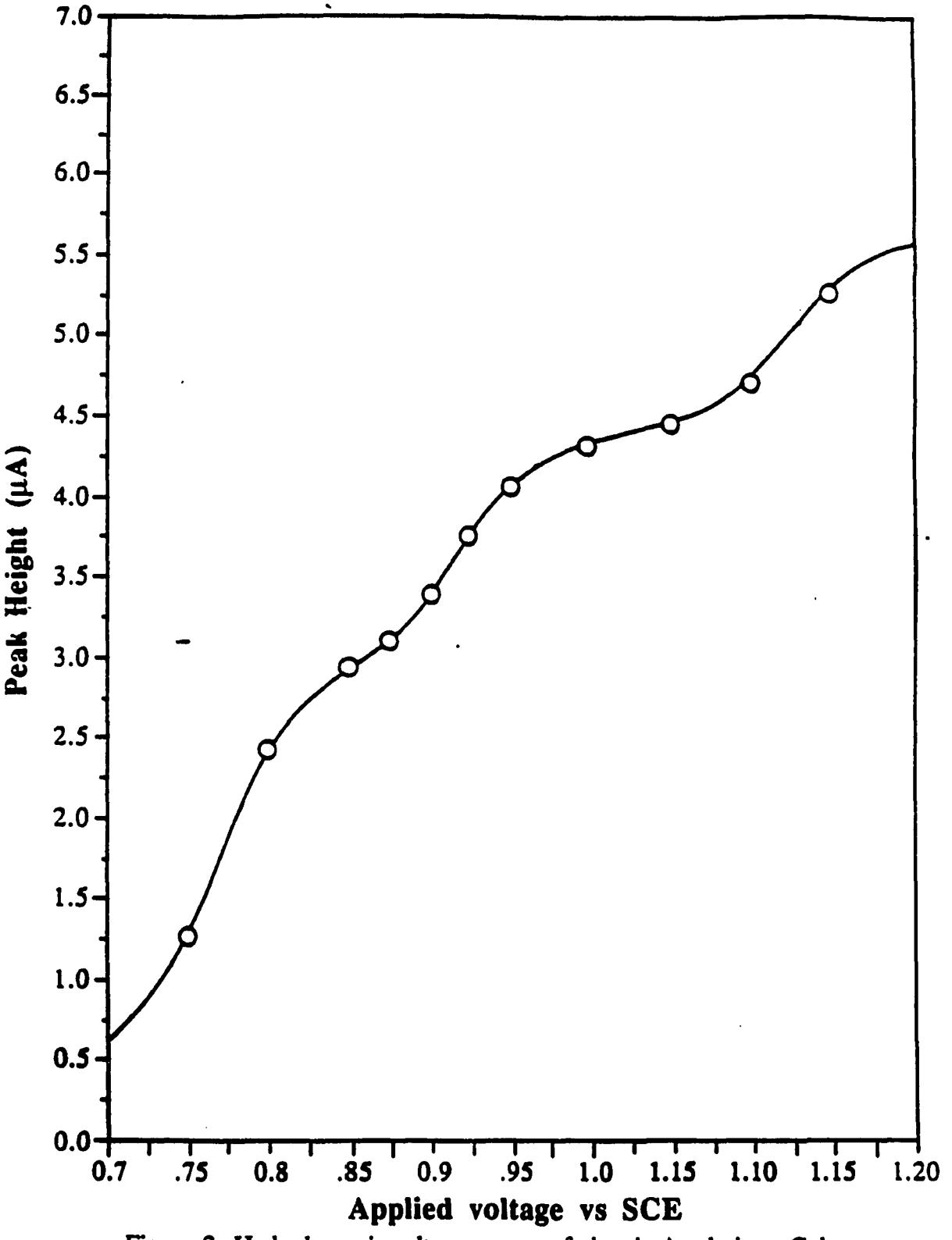

Figure 2. Hydrodynamic voltammogram of vitamin A palmitate. Column: 150 x 4.6 mm I.D. Altex C-8 (10 μm). Mobile phase: acetonitrile - isopropanol-aq 0.025M $NaClO_4$ (45+45+10, v/v). Flow rate: 2.0 mL/min. Sample size: 20 μL aliquot injected of 275 μg/mL standard vitamin A palmitate in mobile phase.

Role of mobile phase composition

The mobile phase composition was systematically varied to optimize analytical sensitivity and steady-state background current. The influence of mobile phase composition on background and sensitivity is shown in Figures 3 and 4 respectively. As shown in Figure 3, a rather dramatic decrease in background is observed as the isopropanol content increases. This is presumably due to the decreasing dielectric constant of the eluent mixture. On the other hand, the effect of of eluent composition on the peak height sensitivity for vitamin A is shown in Figure 4. As expected, the peak height decreased with increasing isopropanol proportion, which can be attributed in part to elongated retention times and broader peaks. The maximum isopropanol proportion that was feasible was 45%, above which the selectivity dramatically decreased.

Influence of electrolyte concentration

No current was recorded in absence of sodium perchlorate. At concentrations of electrolyte below 0.025M, the peak height increased with electrolyte concentration. Above this concentration, a plateau and even a small decrease in response is seen (Figure 5). At higher concentrations the increase in viscosity of the media interfers with the process of diffusion hence resulting in slight decrease in current. A sodium perchlorate concentration of 0.025M was selected for all further HPLC separations.

Effect of Flow rate

Dependence of peak current on mobile phase flow rate is depicted in Figure 6. Since the process of electrochemical reduction is diffusion-controlled, the initial increase in flow rate causes peak current to rise rapidly and then more slowly until a plateau is reached. When replotted on a log-log scale, the data in Figure 6 give a straight line with a slope of 0.39.

Calibration curve (response vs concentration)

The response of the ECD to vitamin A palmitate exhibits a wide range of linearity from 550 pg to 5.5 µg. A typical working calibration curve used to determine vitamin levels is shown in Fig. 7. The curve is linear in the range of 3.5 to 35 µg/mL and passes

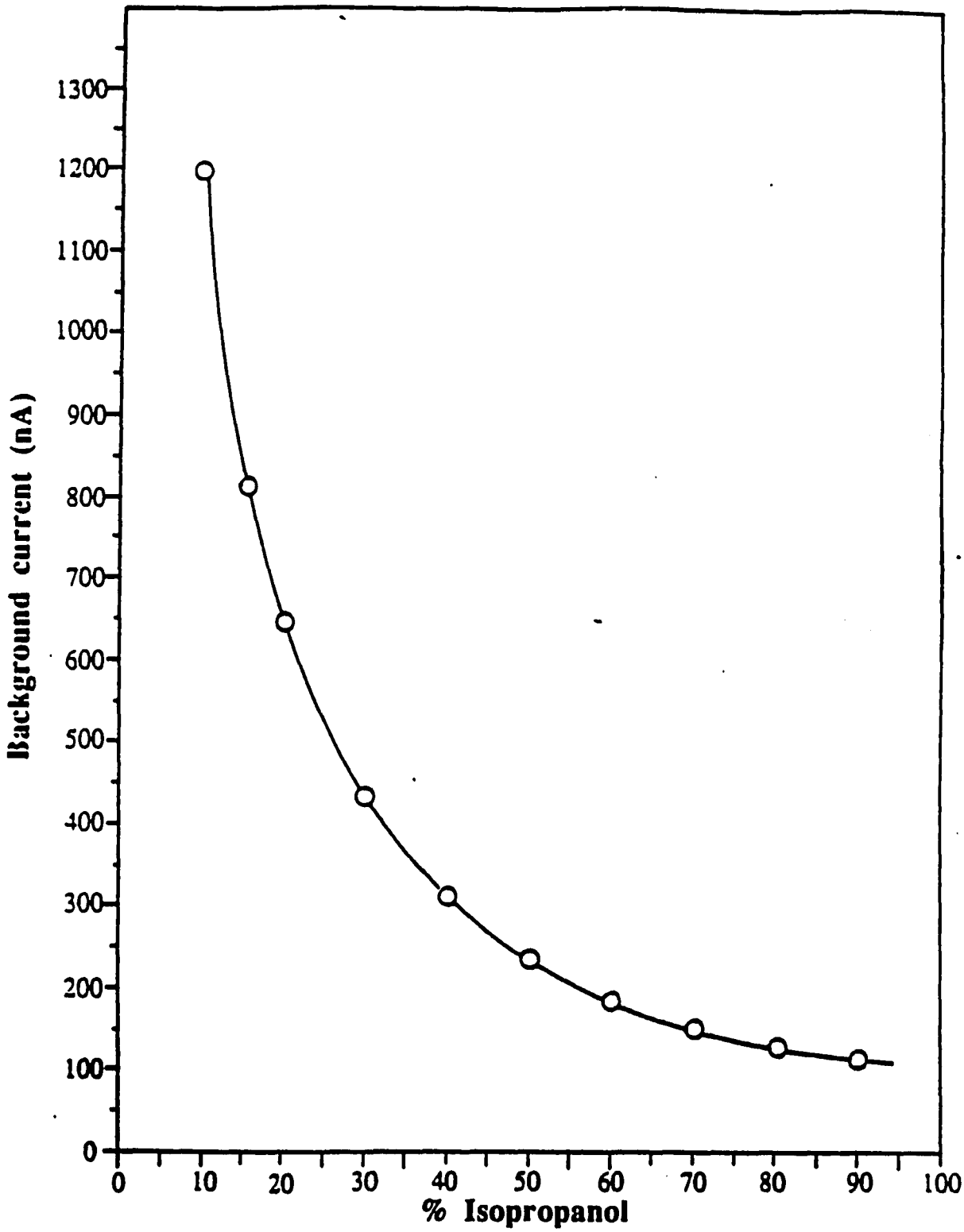

Figure 3. Background current as a function of isopropanol content of mobile phase. Chromatographic conditions: Column, 150 x 4.6 ID mm Altex C-8 (10 μm); mobile phase, prepared from appropiate volumes of isopropanol and stock solution of 0.025 M $NaClO_4$-acetonitrile (18/82, v/v); flow rate, 2.0 mL/min; applied potential, 1.2 V *vs.* SCE.

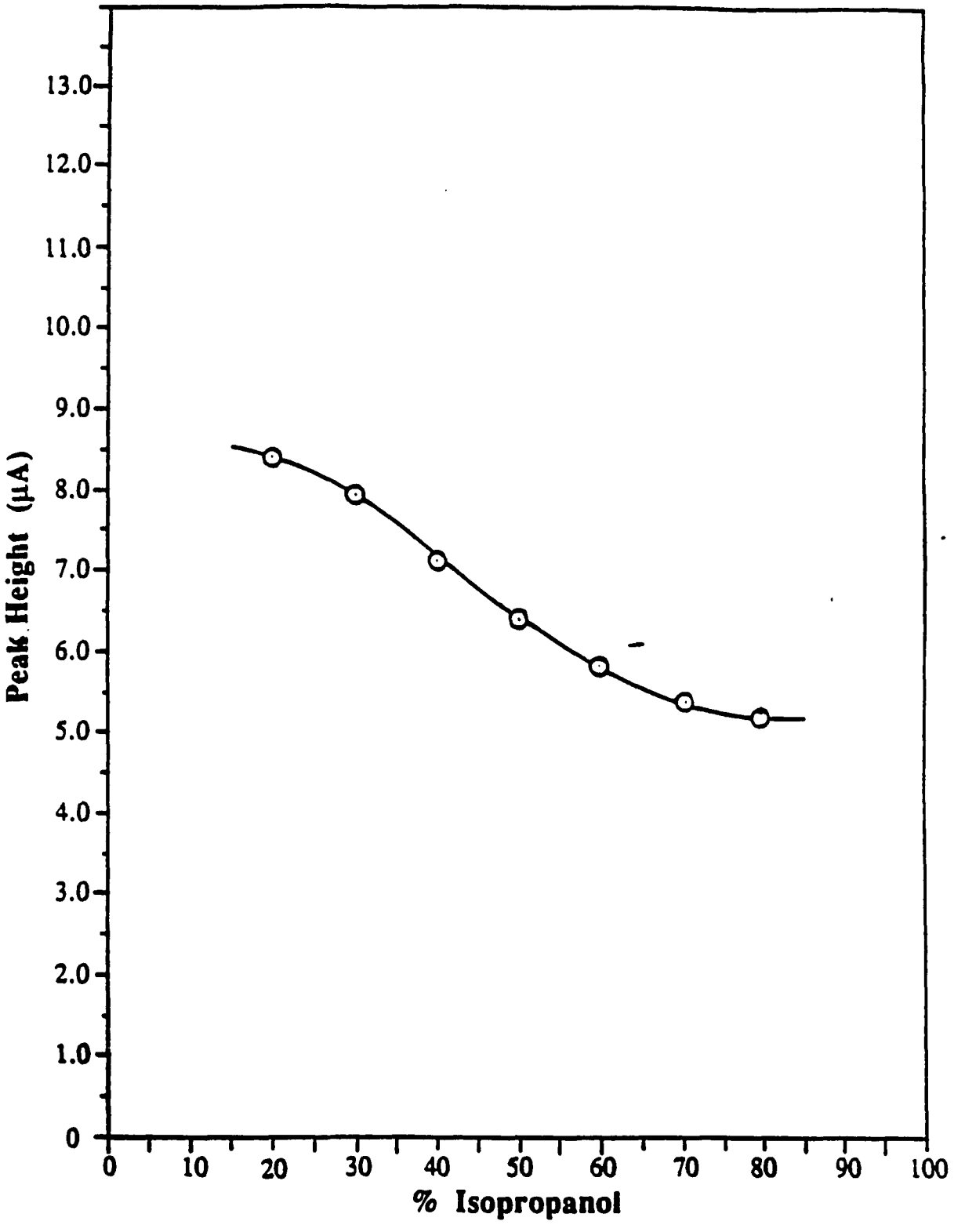

Figure 4. Effect of isopropanol content on the magnitude of the vitamin A palmitate reduction signal. Chromatographic conditions as in Figure 2.

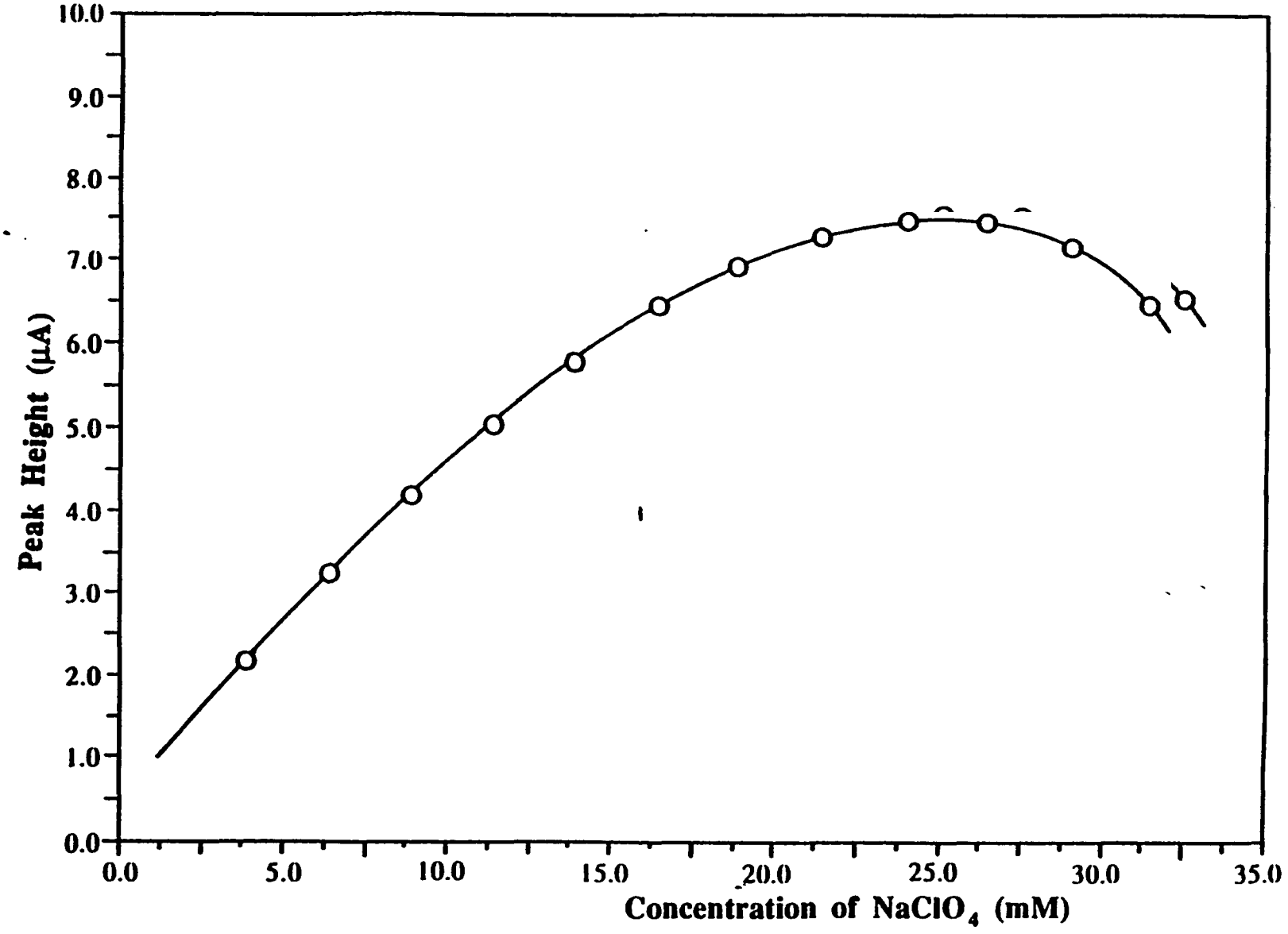

Figure 5. Influence of the concentration of $NaClO_4$ on the reduction of vitamin A palmitate. Conditions: stationary phase, Altex C-8 (10 μm); mobile phase, acetonitrile-isopropanol-water (45+45+10,v/v); flow rate, 2.0 mL/min; applied potential, 1.2 V *vs* SCE.

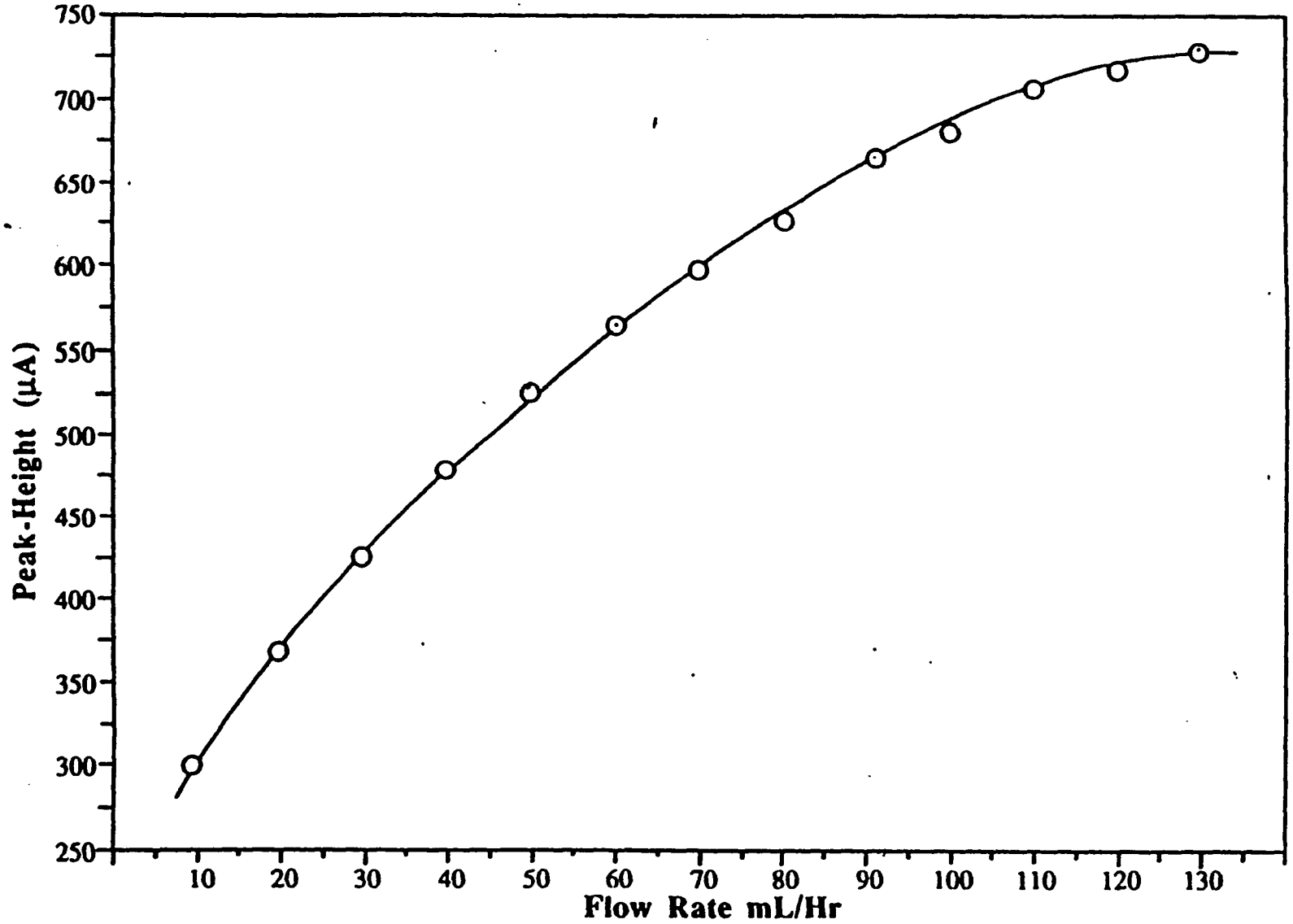

Figure 6. Dependence of peak current on flow rate. Chromatographic conditions as in Figure 2.

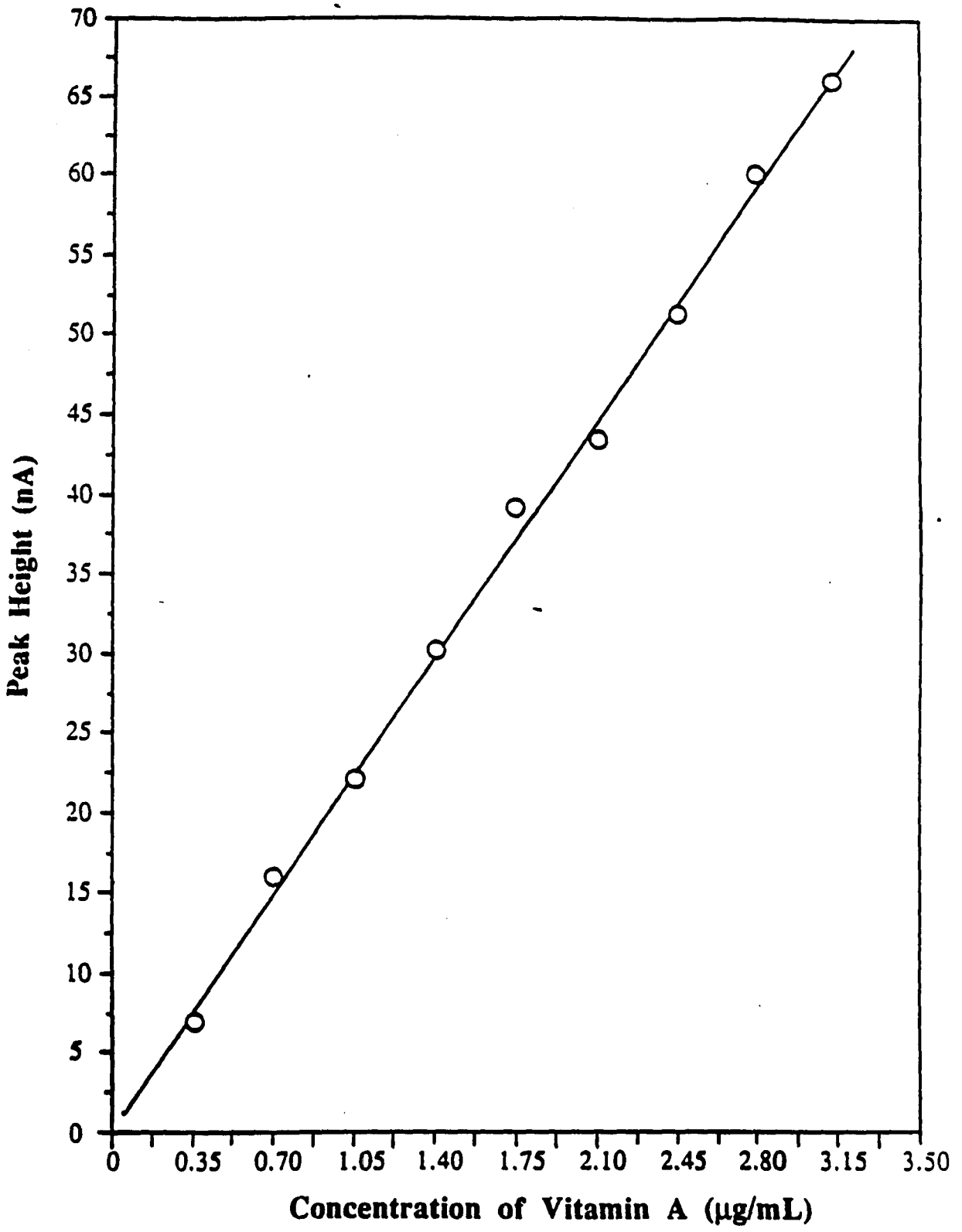

Figure 7. Detector response curve for vitamin A palmitate. Chromatographic and detector conditions: column, 150 x 4.6 mm I.D. mm Altex C-8 (10 µm); mobile phase, acetonitrile-isopropanol-aq. $NaClO_4$ (45+45+10, v/v); flow rate, 2.0 mL/min; applied potential, -1.1 V *vs.* SCE.

within the experimental errors through the origin. Based upon a signal/noise ratio of 3:1 and a background current of 90 pA, the limit of detection is approximately 275 pg.

Optimization of extraction conditions

To develop a quantitative method of extraction of vitamin A , the first step was to optimize the extraction parameters; temperature, pressure and time of equilibration. The influence of pressure on extraction efficiency at 45 ^{0}C and 60 ^{0}C was evaluated by extracting 1.0 g samples of Chromosorb W spiked with 50 μg of vitamin A at several pressures in the range of 5000 to 8000 psi. The dependence of pressure on percentage recovery for each temperature is illustrated by two curves in Figure 8. From curve (A) at 60 ^{0}C, it may be seen that efficiency rises steeply in the region of 5000 to 7500 psi and reaches a plateau around 8000 psi. When the temperature is lowered to 45 ^{0}C, a sigmoidal shape curve (B) is observed over 5000 to 7500 psi range with a limiting efficiency far below 90%. The optimum extraction time was also studied. As shown in table 1, the extraction is complete within 15 minutes, with essentially no change up to 30 minutes.

For maximum sensitivity, an equilibration time of 15 minutes at 8000 psi and 60 ^{0}C was chosen. Under these extraction conditions, a chromatogram of an extract obtained by means of supercritical fluid extraction of a Chromosorb W spiked with 25 μg/g with vitamin A palmitate is shown in Figure 9. Chromatographic analysis shows only one peak at 10 min for vitamin A with a mean percentage recovery of 94% and a coefficient of variation of 3.1% (n=3).

Application of the batch SCFE-HPLC-ECD method on fortified samples

The recoveries for the various cereal ground cereals fortified at 4 levels ranging from 10 to 125 μg/g are presented in Table 2. Efficiencies in the Table 2 represent the average of three extractions. Overall, as demonstrated in Table 1, extraction recoveries remained high over the entire range of concentrations examined. The average recovery of vitamin A palmitate over this entire range was 94.6% with a relative standard deviation of (RSD) of 5.2%. The recoveries of retinyl palmitate were not changed significantly with the change of sample matrix from ground cereals such as corn, to either wheat or oat. Figure 10 is a chromatogram of a wheat sample after supercritical fluid extraction, and chromatographic separation by HPLC followed by electrochemical detection. The chromatographic separations were performed with a mobile phase composed of acetonitrile, isopropanol, 0.025M $NaClO_4$, (45/45/10 v/v) and the mobile phase flow rate was maintained at 2 mL/min. This chromatogram clearly demonstrates that interferences

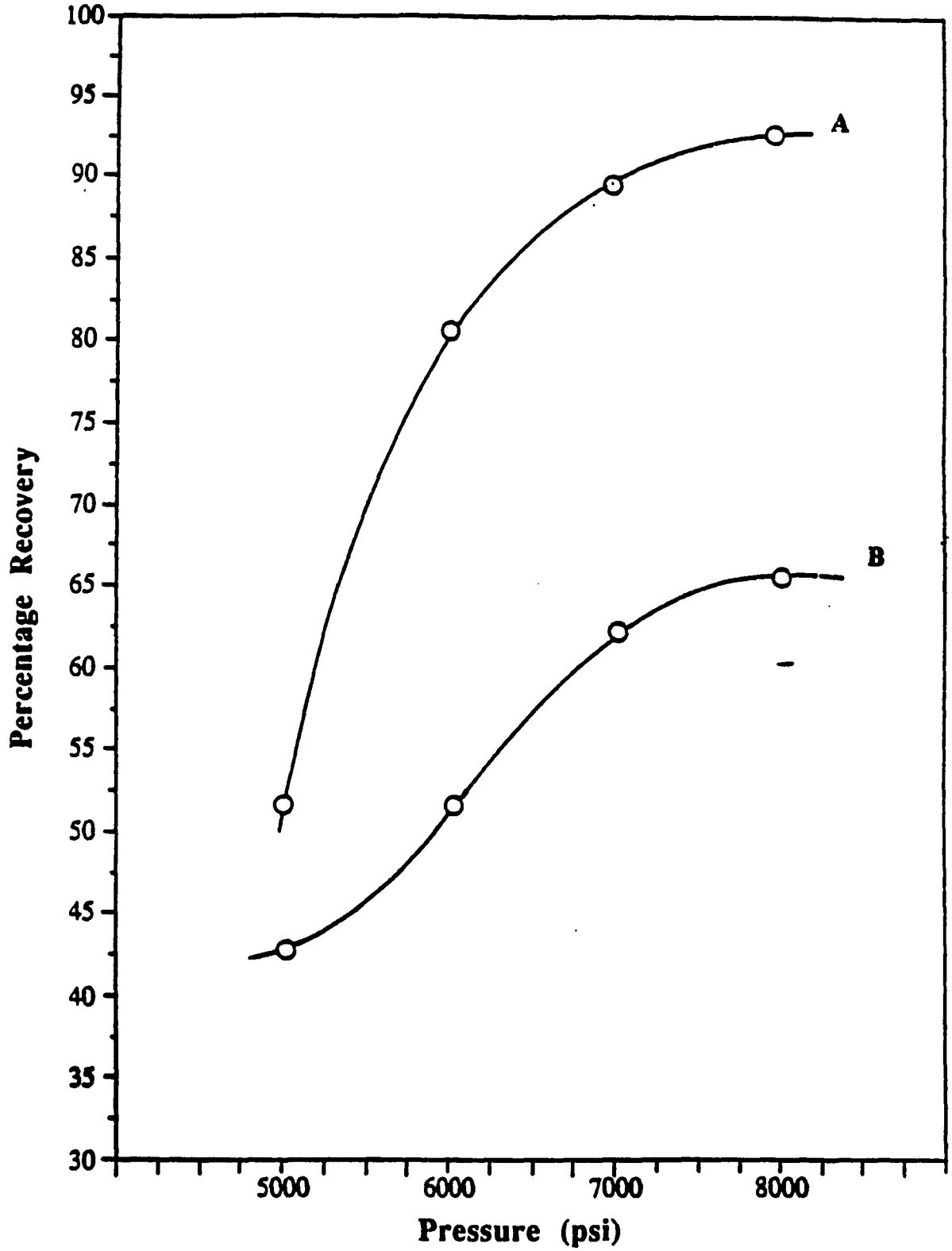

Figure 8. Effect of pressure on extraction efficiency at (A) 60 ^{0}C and (B) 45 ^{0}C. Equilibration time in each case was 20 min. Samples, one gram samples of Chromosorb W spiked with 50 µg of vitamin A palmitate were extracted at each pressure. Chromatographic analysis conditions as in Figure 2.

Table 1. Effect of equilibration time for Vitamin A palmitate* at 8000 psi and 60 ^{0}C.

Time, min	Average Recovery, %	RSD (n=3)
5	74	3.7
10	78	4.1
15	93	3.3
20	91	3.0
30	93	3.4

* Fortification level 50 μg/g Chromosorb W.

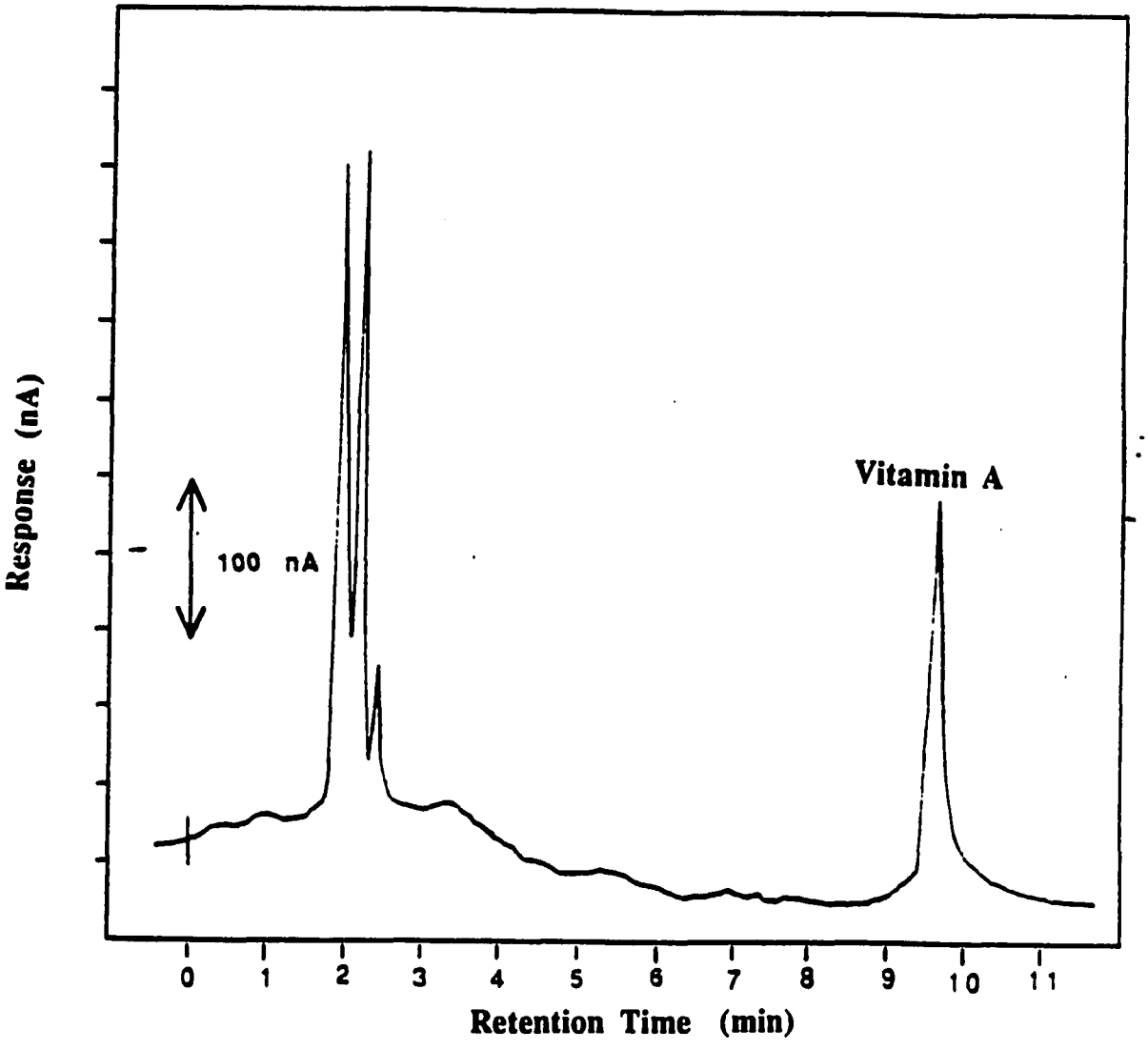

Figure 9. Typical chromatogram of vitamin A standard recovered from a solid phase cartridge by using SCFE sample preparation. Column: 150 x 4.6 mm I.D. Altex C-8 (10 µm). Mobile phase: acetonitrile-isopropanol-aq 0.025 M $NaClO_4$ (45+45+10, v/v). Detector: carbon-based working electrode, applied potential 1.2 V *vs* SCE. Sample, cartridge spiked with 50 µg vitamin A palmitate/ g Celite. SFE at 8000 psi CO_2, 60° C, 20 min.

Table 2. Recovery of retinyl palmitate from fortified cereals.

Cereal	Fortification level (μg/g)	Average Recovery (%)	RSD (n=3)
Corn			
	10	91	6.4
	25	93	6.1
	50	95	5.9
	125	102	5.6
Wheat			
	10	92	3.8
	25	90	3.2
	50	102	3.4
	125	94	2.5
Oat	10	92	5.1
	25	103	4.7
	50	90	4.1
	125	93	4.6

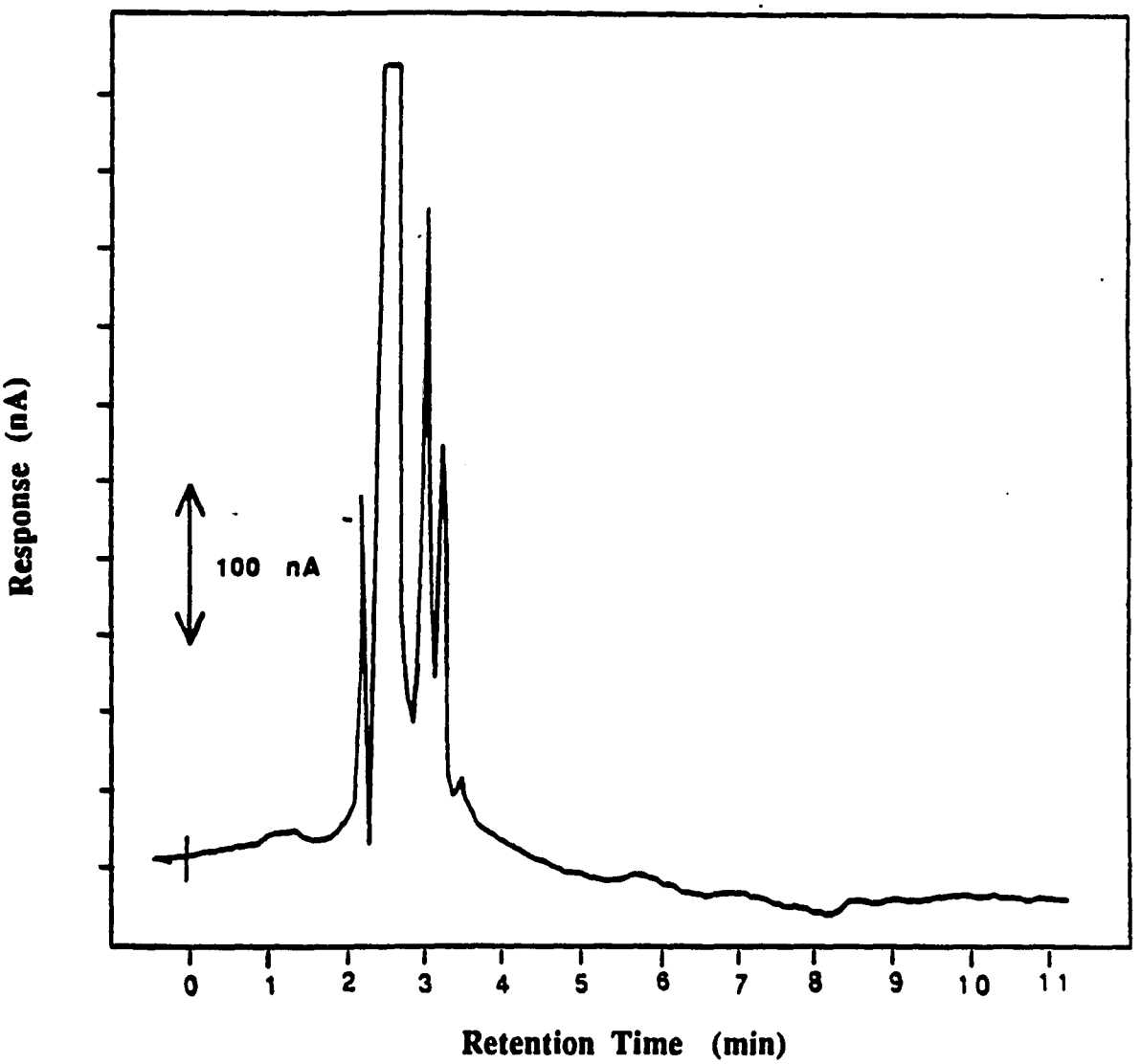

Figure 10. Typical chromatogram of a supercritical fluid extract of an unfortified wheat sample. Column: 150 x 4.6 mm I.D. Altex C-8 (10 μm). Mobile phase: acetonitrile-isopropanol-aq. 0.025 M $NaClO_4$ (45+45+10, v/v). Detector: carbon-based working electrode, applied potential 1.2 V vs SCE. SFE at 8000 psi CO_2, 60° C, 20 min.

contributed by natural compounds were minimal. A typical chromatogram obtained from an extract of a spiked corn is presented in Figure 11. The chromatogram illustrates the degree of resolution of vitamin A palmitate from interfering peaks encountered in this study. The single peak appeared at a retention time of less than 10 min. The identity of the vitamin A peak was verified by co-injection of the corresponding standard. The peaks at 2.5 min and 4.0 min were found in chromatograms from all grains fortified with standard vitamin A and correspond to antioxidant additives present in commercial fromulations of vitamin A.

Effect of Lipids Load

The possible interference of excess lipid on the efficiency of the method was examined by adding microgram amounts of vitamin A dissolved in 250 mg of soybean oil to a series of previously analyzed corn samples fortified at 125 μg/g with vitamin A. The results are shown in Table 3. The recovery of vitamin A palmitate was virtually independent of the interference due to the presence of lipid at this fortification level. Throughout this work, over 50 cereal extracts were chromatographed on the same column without a change in retention time for the vitamin A peak, indicating that column deterioration as a result of lipid carry-over was not a problem.

Precision

Table 4 shows the results of precision (SCFE and electrochemical determination) studies performed with wheat fortified in the range of 25-100 μg/g. For daily precision RSDs ranged from 2.3 to 3.6 %. For day-to-day precision, the RSDs ranged from 3.8 to 8.1%.

Application of SCFE-HPLC-ECD methodology to commercial breakfast formulations

The method as described above was applied to the quantitation of retinyl palmitate in five fortified commercial breakfast cereals with label declaration of 25% and 100% USRDA (Table 5). For all samples extracted and quantitated the vitamin A was found to exceed the declared labels by 9.6% to 65%, indicating the vitamin A didnot decompose during the SCFE sample preparation and electrochemical determination. The coefficient of variation of triplicate determinations of the same sample ranged from 1.6 to 4.8%. A typical electrochemical chromatogram of a wheat based extract prepared by SCFE is depicted in Figure 12. As can be seen, retinyl palmitate is well resolved from early eluting excipients. This good resolution and the freedom from complications makes quantitation easy.

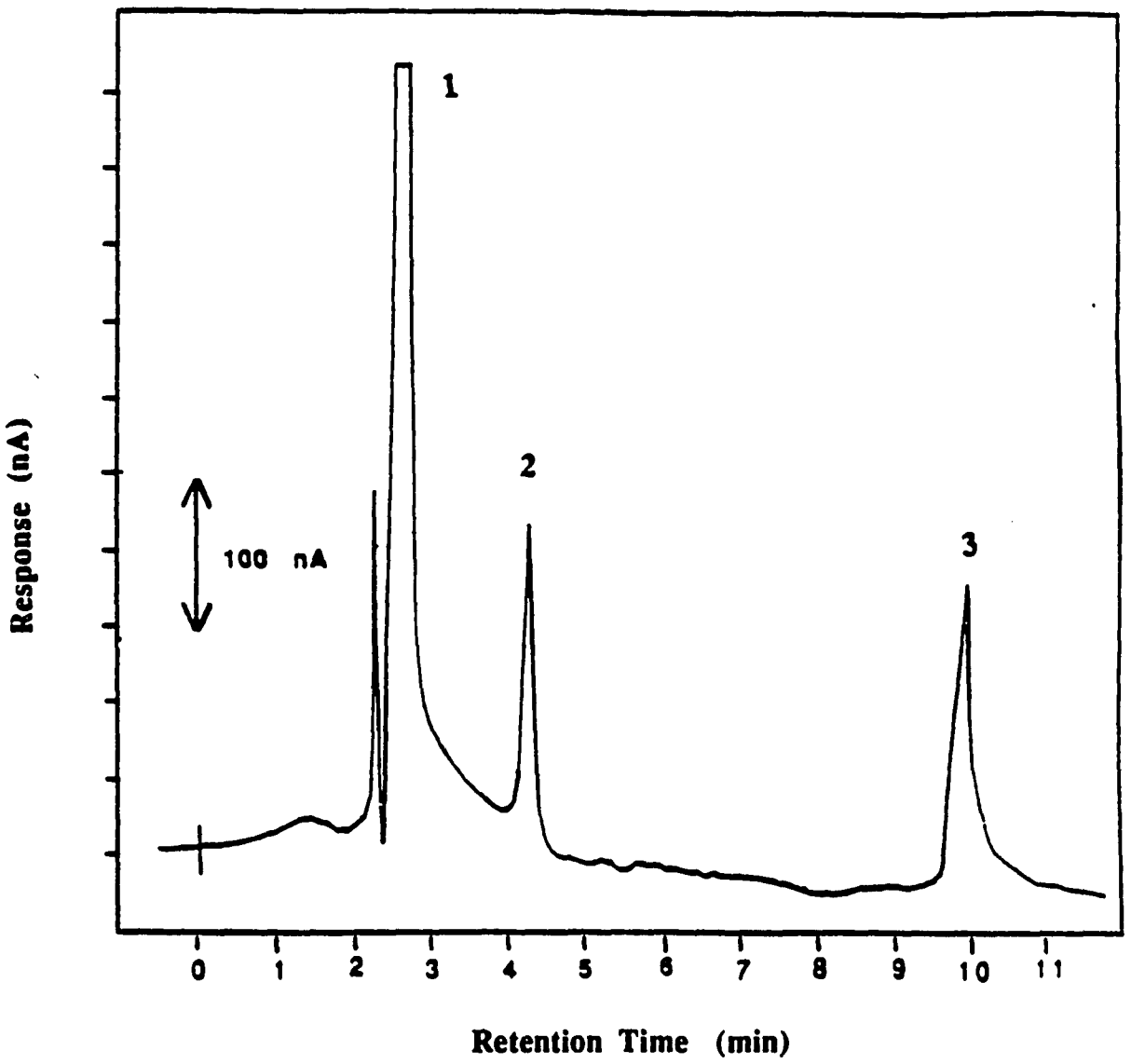

Figure 11. Typical chromatogram of a supercritical fluid extract of a corn sample fortified with vitamin A palmitate prior to sample preparation and chromatographic analysis. Column: 150 x 4.6 mm I.D. Altex C-8 (10 µm). Mobile phase: acetonitrile-isopropanol- aq 0.025 M $NaClO_4$ (45+45+10, v/v). Flow rate was 2.0 mL/min. Detector: carbon-based working electrode, applied potential 1.2 V vs SCE. SFE at 8000 psi CO_2, 60°C, 20 min. Peak identities are as follows: (1), BHT; (2), impurity; (3), vitamin A palmitate.

Table 3. Effect of Lipid Carry Over on Efficiency of Sample Preparation Procedure.

Sample	Vitamin A Added, µg	Initial Sample µg/g	Composite µg/g	Recovery, %
A	10	127	135	99
B	20	129	150	101
C	30	129	149	93
D	40	129	174	103

Average = 99 %
Range = 93 -103 %
RSD = 4.3 %

Table 4. Precision Results For Retinyl Palmitate Assay.

Concentration spiked, µg/g	Mean Found, µg/g	n*	RSD
Daily			
25.0	25.8	3	3.6
50.0	48.8	3	3.0
100.0	102	3	2.3
Between-day			
25.0	24.6	5	8.1
50.0	51.2	4	5.5
100.0	98.6	4	3.8

n* = number of days for the between-day results and the number of samples analyzed on one day for within-day results.

Table 5. Determination of retinyl palmitate in commercially fortified breakfast cereals.

Cereal	Base	% US RDA*	Vitamin A Content, µg/g	RSD (n=3)
Rice Krispies	Rice	25	33	2.1
Special K	Rice	25	39	3.3
Corn Flakes	Corn	25	30	3.2
Raisin Bran	Bran	25	41	4.8
Product 19	Bran	100	110	1.6

* %US RDA 25 = 24.7 µg/g (µg retinyl palmitate/ g cereal)
%US RDA 100 = 98.8 µg/g (µg retinyl palmitate/ g cereal)

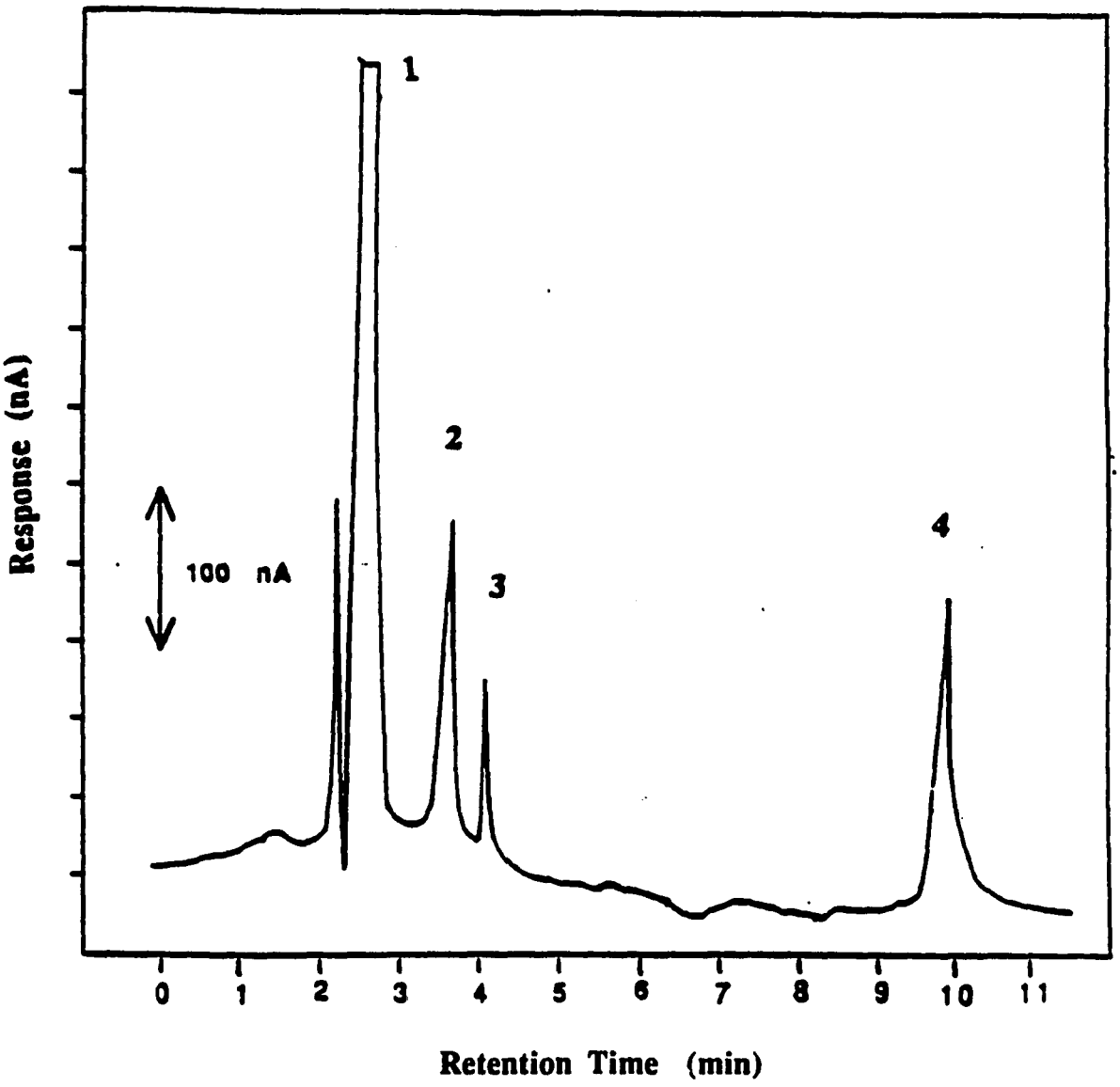

Figure 12. Typical chromatogram of a supercritical fluid extract of a wheat-based ready-to-eat breakfast cereal. Column: 150 x 4.6 mm I.D. Altex C-8 (10 µm). Mobile phase: acetonitrile-isopropanol- aq .025 M $NaClO_4$ (45+45+10,v/v). Flow rate was 2.0 mL/min. Detector: carbon-based working electrode, applied potential 1.2 V vs SCE. SFE at 8000 psi CO_2, 60^0C, 20 min. Peak identities are as follows: (1), BHT; (2), impurity; (3), impurity; (4), vitamin A palmitate.

Conclusion

The combination of SCFE and HPLC-ECD provides a rapid (total sample handling time 50 min), gentle, highly reproducible, single-step, highly efficient and relatively trouble-free method of extraction and selective and sensitive detection procedure for photosensitive and thermally labile analytes such as vitamin A. The method of determination of retinyl palmitate is linear in the concentration range of 10 μg/g to 125 μg/g of cereal. Due to the selectivity of electrochemical detection, the methodology is largely immune to the presence of other interfering extractable materials such as lipids in the sample matrix.

Chapter 7

DETERMINATION OF 1-NITROPYRENE IN DIESEL PARTICULATE EMISSIONS

INTRODUCTION

1-Nitropyrene (1-NP) is of interest both as a specific compound and as a member of an important class of compounds found in emission from diesel engines. 1-NP and its isomers are reported to account for as much as 40% of the Ames assay mutagenic activity of diesel exhaust (1, 2). As one of the predominant nitroarenes in diesel emissions (3, 4), 1-NP serves as a marker for the class of nitroaromatics in these samples. The complexity of diesel particulate matter renders difficult the identification and determination of any specific compound or even groups of compounds, especially those present at relatively low levels. Consequently, following solvent extraction of organics from diesel particulate matter, a cleanup step is required to isolate the nitroarene fraction. Various chromatographic methods have been applied to the determination of specific nitro compounds in this fraction.

In recent work, the procedure for the extraction of 1-NP from diesel samples is usually an 8-24 hour Soxhlet extraction with CH_2Cl_2; the somewhat faster ultrasonic extraction has also been used (5, 6, 7) but recovery of 1-NP from spiked diesel particulate was only 30% at the 1 ppm level and 80% for 10 ppm (7); recovery data were not given by Robbat, et al. (5) or Nelson (6). Cleanup is commonly accomplished by using a silica column with a solvent gradient (8), or a Waters Sep-Pak (4, 5, 9,10). Nitrated polycyclic aromatic hydrocarbons (PAH) have been determined using gas chromatography (GC) with flame ionization (FID) (5, 6, 11-13), electron capture (4, 11), or thermoionic ionization detectors (NPD) (6, 9, 11, 12, 14). Campbell and Lee (15) chemically reduced the nitro group to amino, formed pentafluoropropylamide derivatives, and determined these by capillary GC with electron capture, NPD, FID, and GC/mass spectrometry (MS). Nitrated PAH in diesel exhaust have also been determined using GC with a chemiluminescence detector (Thermo Electron Analyzer), which is highly sensitive and selective (5, 7, 16). Detailed studies of nitro compounds extracted from diesel exhaust particulate matter have often used capillary GC with online MS (9, 13, 14, 17-20), negative ion chemical ionization MS (11, 17), high resolution MS (17, 19), MS/MS (19, 21), in addition to GC/FTIR (17). Karasek, et al. (13, 18) have cautioned that 1-NP can partially decompose to 1-aminopyrene during GC/MS analysis, both on and after the column. They developed a compensation technique using deuterium-labelled 1-NP-d_9, enabling quantitative work to be done.

High performance liquid chromatography (HPLC) has also been applied to nitroarene determination determination in both normal and reversed phase modes. Silica columns (6) as well as a specially synthesized pyrene-butyric acid amide-bonded phase microbore column (22) have been used with UV detection. The latter column was used with a C_{18} reversed phase column with column switching to isolate and quantitate 1-NP. Post-column conversion of nitro compounds to amino analogs followed by fluorescence detection (9, 20) has also been used. Reductive mode electrochemical detection of nitroarenes has been applied with glassy carbon electrodes (GCE) (10, 23) and gold amalgam electrodes (20), following reversed-phase HPLC separation. The linear range of GCE is reported to be about 10^3, with a sensitivity of 10-100 pg (10). Hydrodynamic voltammograms (HDVs) can be an aid to qualitative identification of nitro compounds (10). The electrochemical detector is sensitive to quinones, and anthraquinone and 1,4-naphthoquinone which were detected in diesel exhaust particulate matter (10).

We have developed a rapid procedure for 1-NP based on the supercritical fluid extraction (SCFE) of the compound followed by HPLC with a reductive mode ECD using a silver electrode. The extract, which is obtained in 20 min, is trapped by depressurization of the SCF CO_2 solvent across a short silica-packed tube, and eluted with a small volume of CH_2Cl_2/CH_3COCH_3. Solvent is evaporated, the residue taken up in the HPLC eluent, and 1-NP separated from other electrochemically reducible species in 5.5 min. Results of the determination of 1-NP in the National Bureau of Standards (NBS) Standard Reference Material (SRM) 1650 (Diesel Particulate Matter) using this method, are in excellent agreement with the NBS certified value.

EXPERIMENTAL

Reagents

1-Nitropyrene (Gold label grade) was purchased from Aldrich Chemical Co., (Milwaukee, WI). HPLC grade acetonitrile, acetone, and methylene chloride were obtained from J. T. Baker (Phillipsburg, PA). In-house glass distilled water was used throughout the study.

Apparatus

The chromatograph was built from components. A Varian 8500 syringe pump provided pulse free flow of eluents. Samples were injected with a Rheodyne 7125 sampling

valve. Sample solutions were placed in a 4 mL Reacti-Vial, degassed with a stream of helium or nitrogen, and pneumatically flushed through the 20 µL sample loop to avoid any exposure to air. Oxygen is detected by reductive-mode ECD at voltages more negative than about -0.2 V vs SCE. The column was 15 cm x 3.9 mm id, 10 µm µBondapak C_{18} (Waters Associates, Milford, MA). The mobile phase conditions, 60/40 (v/v) acetonitrile/ 0.025 M aq. $NaClO_4$ at 2 mL per min, were established experimentally to separate the 1-NP peak cleanly from the other peaks detected, and to minimize analysis time. Eluent was degassed by rapid bubbling of He or N_2 through a porous steel diffuser for 15 min. ECD detection was accomplished with laboratory constructed silver based thin layer cell (Appendix 1).

GC/MS

GC peak identities were confirmed using a Hewlett-Packard Model 5988A GC/ quadrupole MS with Hewlett-Packard 1000 data system. A 30 m x 0.25 mm id bonded phase fused silica capillary column, J & W DB-5 (J & W Scientific, Folsom, CA) was used. An initial, 5 min temperature of 150 °C was followed by a 10 °C/min temperature program to 280 °C, with a 40 min final hold at 280 °C.

Procedure

100 mg of diesel samples (NIST, SRM 1650) were loaded into a 6 in. x 3/8 in. extraction chamber and held in place with glass wool plugs. The extraction chamber was assembled into the extraction manifold (Chapter 2), brought to system temperature, and the system flushed for a minute with low pressure CO_2. The exit valve was then closed and the system pressurized to the extraction pressure. After a 15 or 20 min equilibration, the exit valve was carefully and partially opened to allow the extract to be collected. The depressurization occurs across a 6 in. x 1/8 in. o.d. ss tube packed with silica gel attached directly to the exit valve. The solubility of the extract in subcritical pressure CO_2 is essentially zero; the silica acts probably as much as a physical filter as an adsorbent. The silica was removed and washed with methylene chloride-acetone (50/50, v/v); the extraction chamber and exit valve were rinsed with the same. The combined volume of eluate and rinsings was 30 mL. The solution was evaporated nearly to dryness in a rotary evaporator and remaining solvent evaporated in a stream of N_2. The residue was taken up in 10 ml mobile phase for HPLC. For GC and GC/MS 2 µL aliquots were removed from the rotary evaporator flask before final evaporation.

RESULTS AND DISCUSSION

In Figure 1 is shown the hydrodynamic voltammogram (HDV) of 1-NP. The plateau voltage, -1.1 V vs SCE, was used for HPLC quantitation. The $(E_{3/4}-E_{1/4})$ value, 130 mV, indicates the 2-electron reduction is irreversible (24). The detector noise level at the working potential is 75 pA. The minimum detectable quantity, i.e., that weight of 1-NP injected producing a peak height three times the noise level, is 197 pg. The response is linear to at least 7.6 μg 1-NP injected, i.e. over at least a range of 4×10^4 ; the upper bound is limited by solubility of 1-NP in the partially aqueous mobile phase.

EXTRACTION

In order to establish the optimum conditions of extraction for 1-NP, a 2.5 g sample of 100/120 mesh Chromsorb W was spiked with 2 μg of 1-NP and extracted at four pressures in the range of 5000 to 8000 psi at both 45 ^{0}C and 65 ^{0}C for 20 min. The percentage recoveries are shown in Tables 1 and 2 for 45 ^{0}C and 65 ^{0}C , respectively. As can be seen, recoveries increase with increase in pressure at both temperatures but the effect is more pronounced at 65 ^{0}C. Table 3 shows that percentage recovery of 1-NP reaches the maximum level within 20 min., after which no significant changes in percentage recoveries were observed. For all further work, extractions were performed at 8000 psi and 65 ^{0}C for a period of 20 min. To test the efficacy of these conditions, supercritical fluid extractions were carried out on four samples of Chromosorb W spiked at four different concentration levels of 1-NP. The results are summarized in Table 4. Under these conditions, the average percentage recovery was 93.6 ± 3.3%.

Application to diesel samples

To test the overall method, we analyzed the NIST SRM 1650 for 1-NP using the extraction and determination method described in the experimental section. A typical chromatogram of an extract obtained by SCFE is shown Fig. 2. Confirmation of the identity was carried out in several ways. First, the HPLC peak was collected and analyzed by GC/MS in the selected ion monitoring mode (SIMS) at m/z =247 (molecular ion, M), 217 (M-NO), 201 ($M-NO_2$), and 189 ($M-CNO_2$) (19). The GC retention time matched that of pure 1-NP, and distribution of the ions monitored was similar to those in the mass spectrum of the known 1-NP peak. Second, the SRM 1650 was spiked with 2 μg/g of

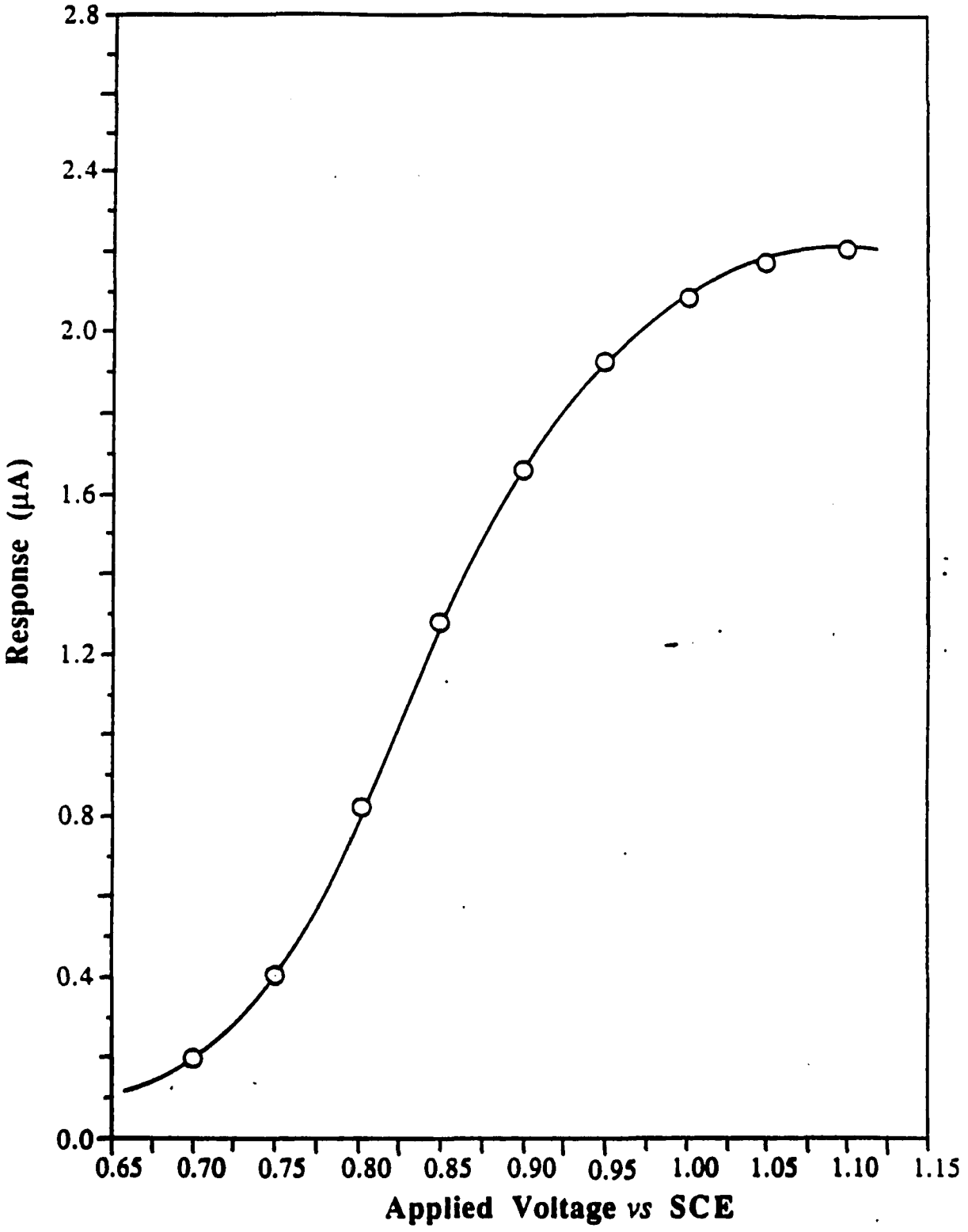

Figure 1. Hydrodynamic voltammogram of 1-nitropyrene on silver electrode vs. SCE. Chromatographic conditions: column, 150 x 3.9 mm I.D. μBondapak C-18 (10 μm); mobile phase composition, 60/40 (v/v) acetonitrile-aqueous .025 M $NaClO_4$; flow rate, 2.0 mL/min; sample size, 0.44 μg 1-nitropyrene per 20 μl mobile phase injected at each potential setting.

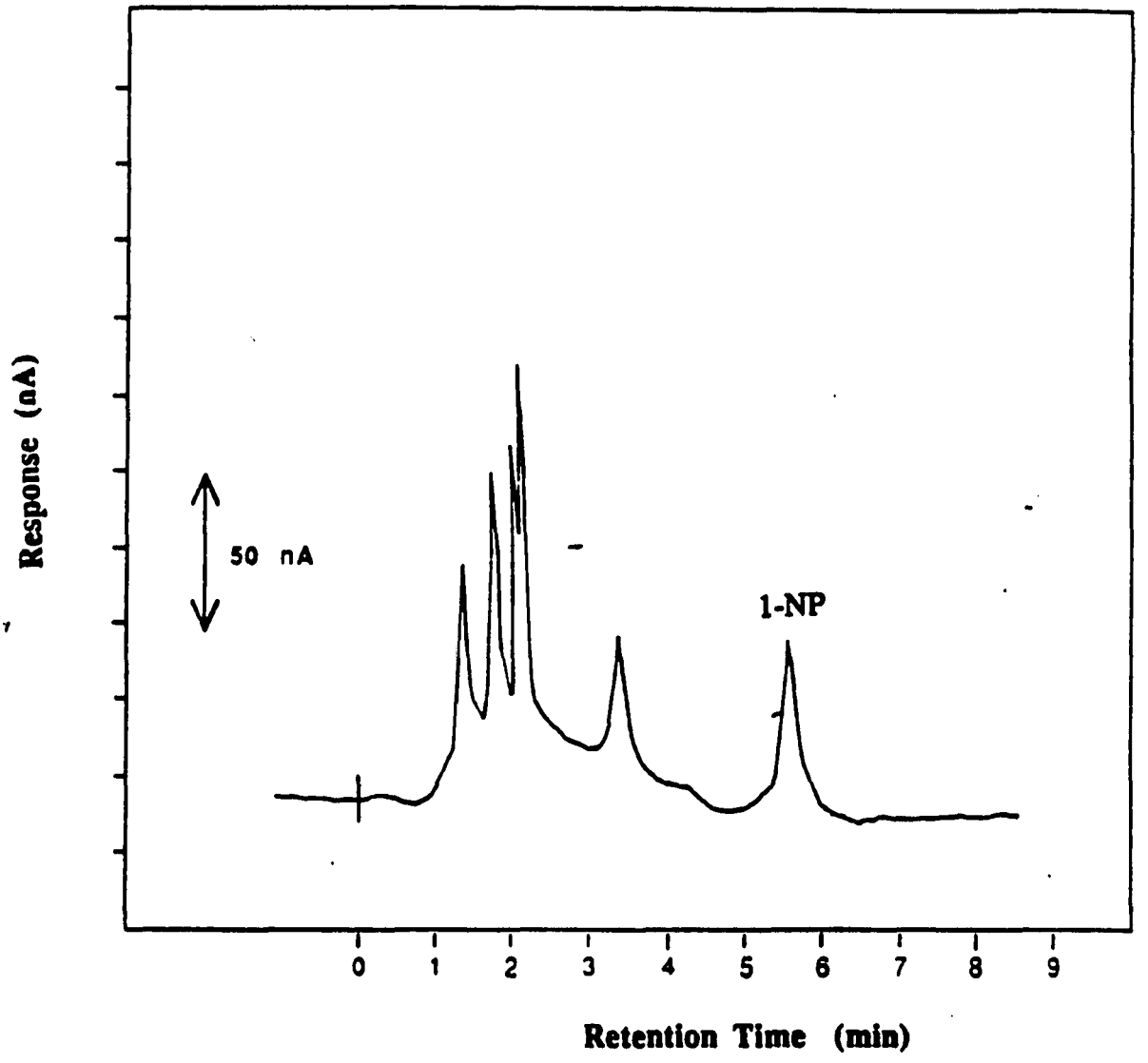

Figure 2. LCEC chromatogram of a high pressure supercritical-fluid extract of NBS SRM 1650 on a 150 x 3.9 I.D. mm μBondapak column using a mobile phase comprised of 60/40 (v/v) acetonitrile / 0.025 M aq $NaClO_4$ at a flow rate of 2.0 mL/min. Extraction conditions: SFE with 8000 psi CO_2, 65°C, 20 min.

Table1. Effect of Pressure on the extraction of 1-NP from ChromosorbW*

Pressure (psi)	Average Recovery (%)	RSD (n=3)
8000	91	4.8
7000	82	5.4
6000	67	5.9
5000	49	6.4

* Temperature of extraction = 65 ^{0}C
Time of equilibration = 20 min.
Level of fortification of 1-NP = 2 μg/2.5 g Chromosorb W

Table 2. Effect of Pressure on the extraction of 1-NP from Chromosorb W*

Pressure (psi)	Average Recovery (%)	RSD (n=3)
8000	74	5.5
7000	52	5.1
6000	39	4.9
5000	22	6.2

* Temperature of extraction = 45 ^{0}C
Time of equilibration = 20 min.
Level of fortification of 1-NP = 2 μg/2.5 g Chromosorb W

Table 3. Effect of Time of Equilibration on the extraction of 1-NP*

Time of Equilibration (min)	Average Recovery (%)	RSD (n=3)
5	60	3.8
10	73	3.6
15	84	4.1
20	91	3.1

* Temperature of extraction = 65 ^{0}C
Pressure of extraction = 8000 psi
Level of fortification of 1-NP = 2 µg/2.5 g Chromosorb W

Table 4. Recovery of 1-NP from Chromosorb W+

Spiking level (μg/g)	Recovery (% ± RSD)*
75	96.0 ± 2.5
50	94.5 ± 2.8
25	95.0 ± 3.3
1	91.0 ± 4.5

+ Temperature of extraction = 65 ^{0}C
Pressure of extraction = 8000 psi
Equilibration time = 20 min.
* n = 3

1-NP, extracted with supercritical fluid carbon dioxide, and the extract analyzed. A typical chromatogram of an extract of a spiked diesel sample is shown in Figure 3. The LC peak retained its original width and increased in height. The GC/MS in SIMS mode found a peak at the retention time of 1-NP in higher intensity and with the same ion distribution as the unspiked sample.

The third method involved step-wise SCFE at two different pressures. This is one of the advantages of SCFE; the ability to use SCFE at one pressure as clean-up step, and at a second pressure to extract the compound of interest. The results are given in Figure 4. The lower pressure SCFE of the SRM 1650 sample at 5000 psi, 65 °C, and 20 min produced an extract with a GC-total mass spectral ion current (TIC) chromatogram as shown in the upper figure (A). A clean silica trap was installed and the extraction pressure raised to 8000 psi and held for 20 min. at 65 °C. The resulting GC/TIC chromatogram is shown in the lower figure (B). It should be emphasized that no clean-up procedure has been used except the two pressure level SCFE; the chromatograms are of the total extract obtained in each case. The paraffin hump characteristic of oil-derived samples has been completely removed at lower pressure. The SIMS is required to confirm the presence of 1-NP in the extract. In Figures 5 and 6 are the SIMS results at the four ions characteristic of 1-NP; it is clear that no 1-NP is extracted at 5000 psi. However, the compound is clearly present in the 8000 psi extract. Identification of the other peaks in Figure 6 have not been made; the peak just preceding 1-NP could be an isomer of 1-NP. A clean-up procedure will be required to characterize nitro- and oxy-PAHs other than 1-NP that may be present in the sample extract.

The HPLC/ECD analysis of the high- and low- pressure supercritical fluid extracts further substantiates the presence of 1-NP in the higher pressure and its absenece in the lower pressure extracts. The lower (C) and the middle (B) chromatograms in the Figure 7 are the HPLC/ECDs at -1.1V vs. SCE of the lower pressure and high pressure extracts, respectively. No electroactive compound whatsoever is observed in low pressure extract. The early eluting peak in higher pressure extract may be a quinone. The short retention time is consistent with the compound's being polar; nitro compounds elute later, e.g. 1-NP. Earlier SCFE work (Chapter 8) has shown that recoveries of quinones are low at 5000 psi but essentially quantitative at 8000 psi. The upper chromatogram in Figure 7, of the higher pressure extract run with the ECD Ag electrode potential set at -0.5 V vs SCE, has only a single peak. The 1-NP peak disappeared, because the foot of its HDV occurs at about -0.65V. The compound is not 9-Fluorenone another compound certified by the NIST in SRM 1650; the HDV for 9-fluorenone, has a foot at -1.1V, and so would not be detected at -0.5V. 9-fluorenone is, however, tentatively identified the coincidence of GC retention times of a peak in the extract and that of a standard. The mass spectrum

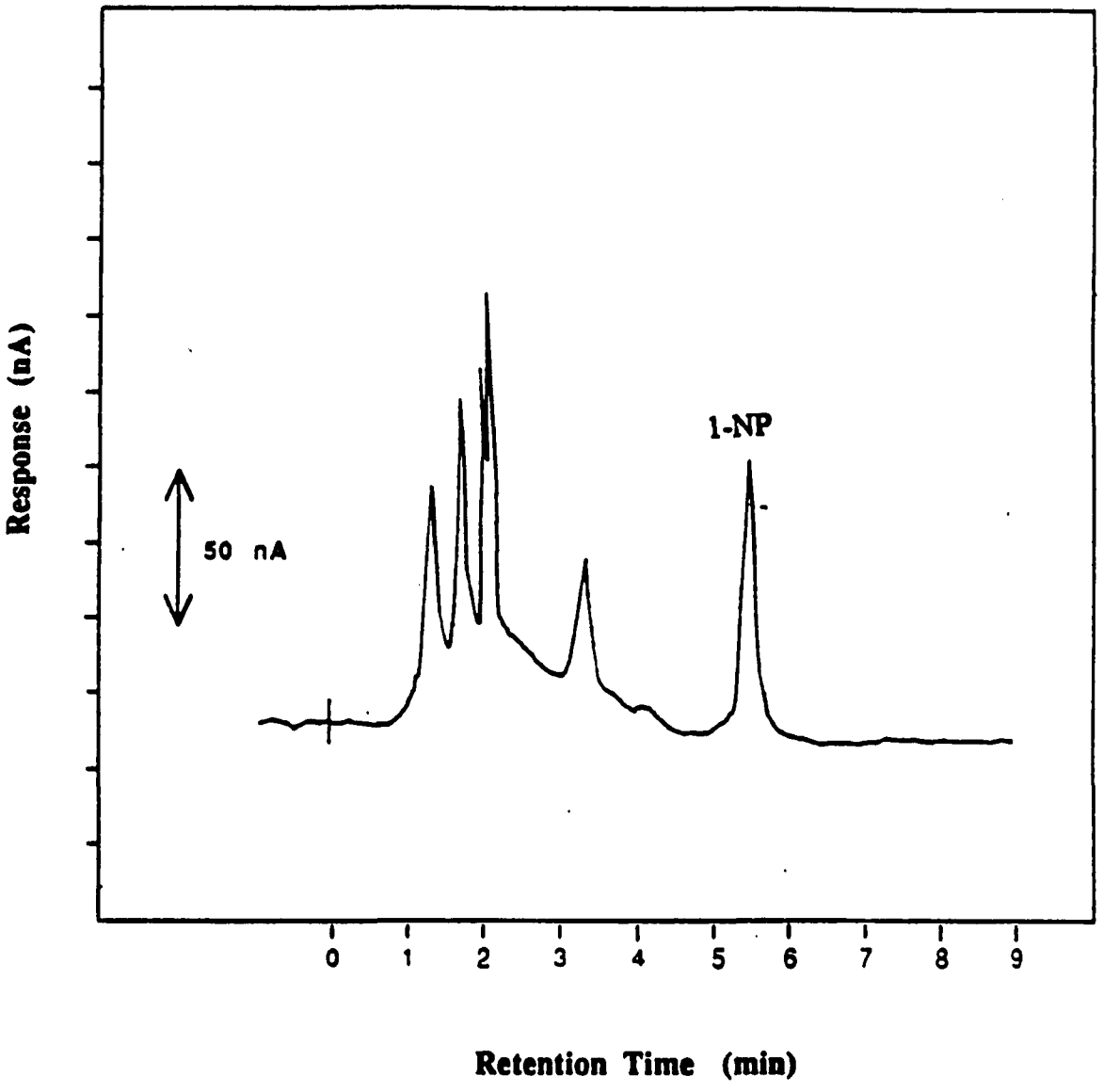

Figure 3. LCEC chromatogram of a high pressure supercritical-fluid extract of NBS SRM 1650 fortified with 1.15 µg of 1-nitropyrene prior to sample preparation and chromatographic analysis. Chromatographic analysis conditions: column, 150 x 3.9 I.D. mm µBondapak (10 µm); mobile phase, acetonitrile - 0.025 M aq $NaClO_4$ (60+40, v/v); flow rate, 2.0 mL/min. Extraction conditions: SFE with 8000 psi CO_2, 65^0 C, 20 min.

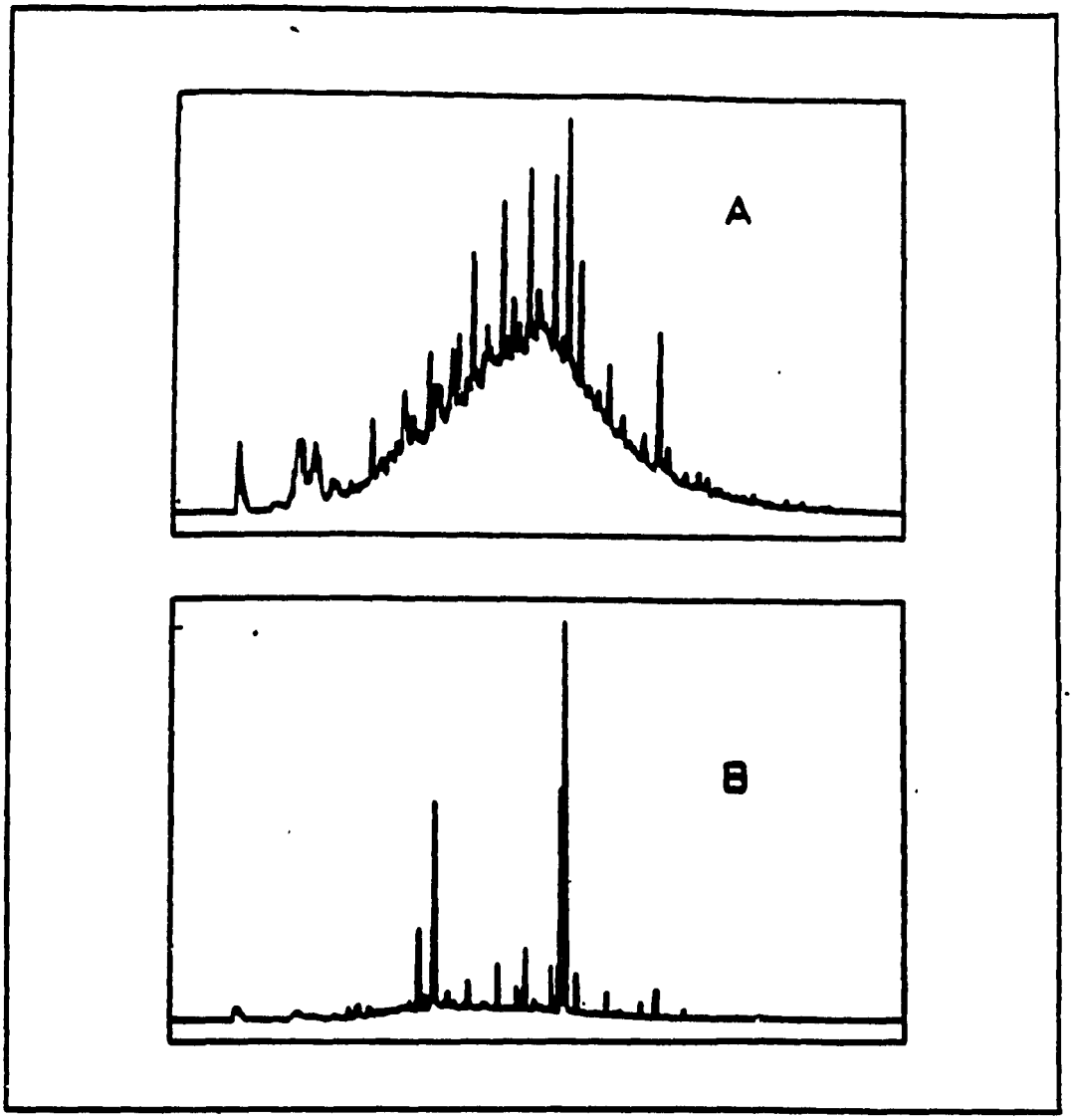

Figure 4. GC/MS total ion chromatograms of (A) low pressure and (B) high pressure supercritical-fluid extracts of NBS SRM 1650. SFE at 5000 psi and 8000 psi for low and high pressure extracts, respectively. A temperature of 65^{0}C and an equilibration time of 20 min used in both extractions. GC conditions: 30 m x 0.025 mm I.D. DB-5 fused-silica capillary column; temperature, 150^{0}C for 2 min, then temperature programmed at 10^0 C/min to 250^0 C; Carrier gas, He, 1 mL/min.

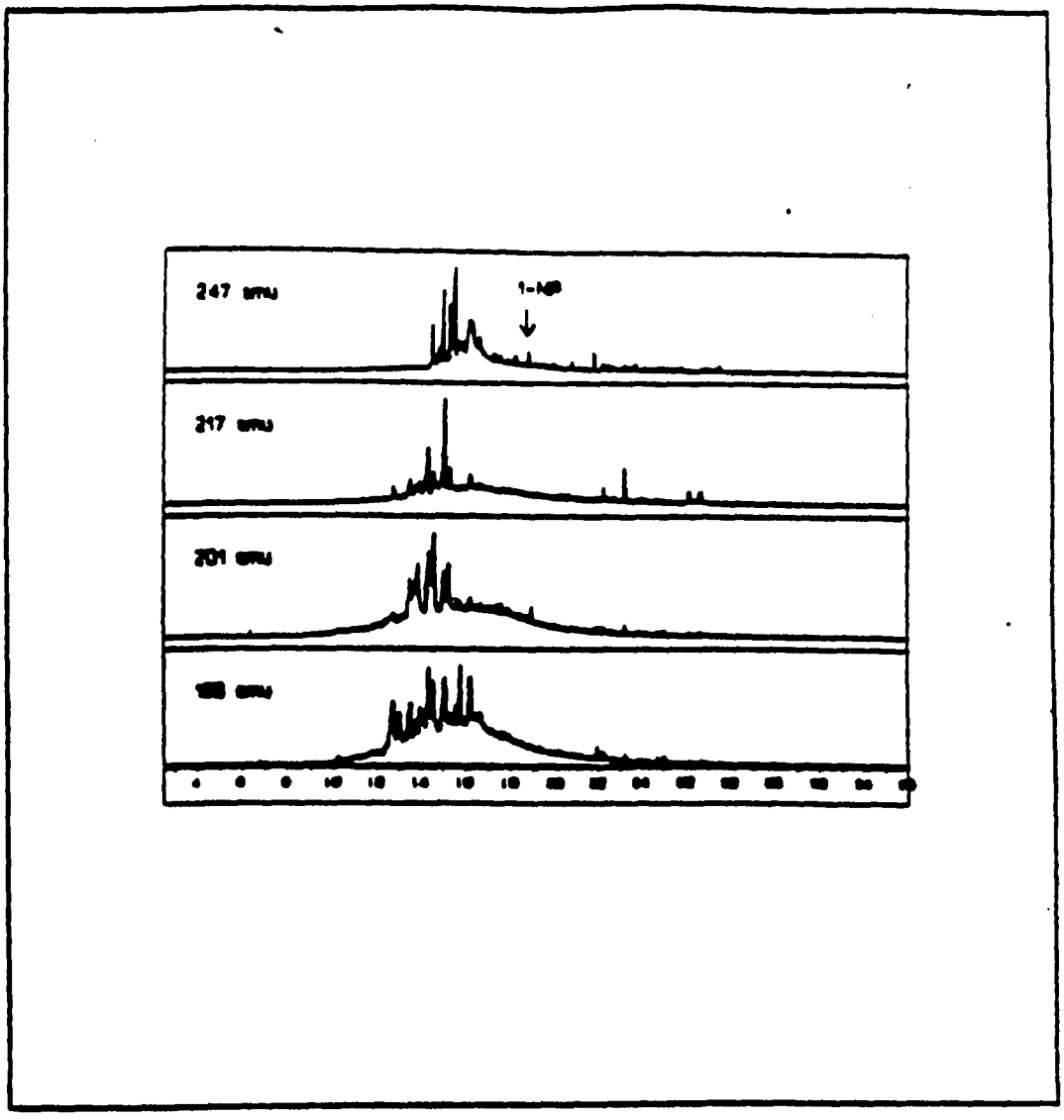

Figure 5. SIMS chromatograms of lower pressure supercritical-fluid extract of NBS SRM 1650. SFE at 5000 psi, 65^{0}C, 20 min. GC conditions: 30 m x 0.025 mm I.D. DB-5 fused-silica capillary column; temperature, 150^{0}C for 2 min, then temperature programmed at 10^{0}C/min to 250^{0}C; Carrier gas, He, 1 mL/min. The arrow indicates the retention time of 1- nitropyrene (1-NP).

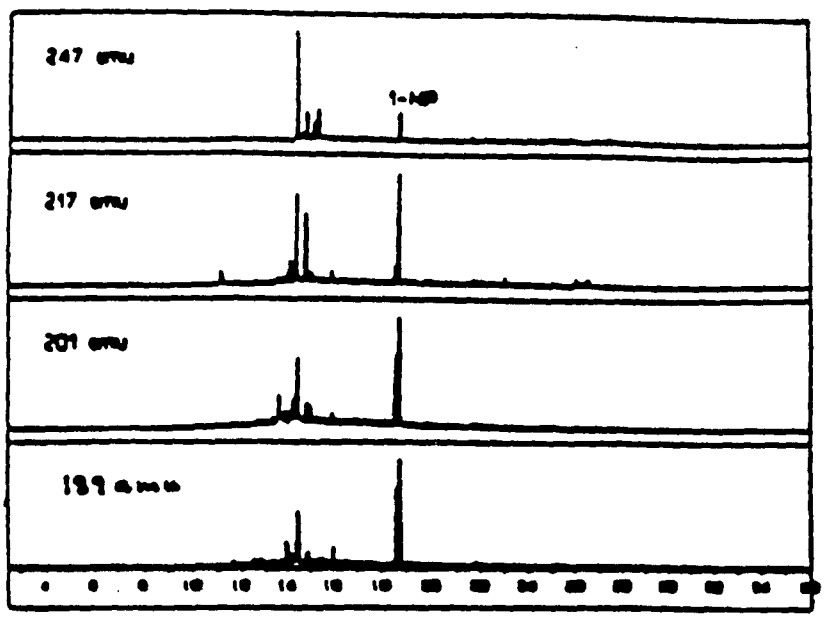

Figure 6. SIMS chromatograms of higher pressure supercritical-fluid extract of two-step SFE of NBS SRM 1650. SFE at 5000 psi, 65^{0}C, 20 min. GC conditions: 30 m x 0.025 mm I.D. DB-5 fused-silica capillary column; temperature, 150^{0}C for 2 min, then temperature programmed at 10^0 C/min to 250^{0}C; Carrier gas, He, 1 mL/min. The arrow indicates the retention time of 1- nitropyrene (1-NP).

intensity is too low to allow reliable identification.

The HPLC/ECD analysis of the high- and low-pressure supercritical fluid extracts further substantiates the presence of 1-NP in the higher pressure and absence in the lower pressure extracts. The lower (C) and middle (B) chromatograms in Figure 7 are the HPLC/ECDs at -1.1V vs SCE of the lower pressure and higher pressure extracts, respectively. No electroactive compound whatsoever is observed in low pressure extract. The early-eluting Quantitation of the 1-NP peak in Figure 2 was carried out by comparison of the peak height with a linear calibration plot obtained by injecting standard solutions of 1-NP dissolved in mobile phase into the LC. Our result based on HPLC-ECD is 19.9 μg 1-NP/g of the diesel particulate sample, in excellent agreement with the NBS certified value, 19 ± 2 μg/g. Quantitation by GC/MS was not attempted because of the thermal breakdown problems noted by Karasek, et al. (13, 18). The deuterated standard was not available to us.

Conclusion

1-Nitropyrene is extracted from a complex matrix like diesel exhaust sample (SRM 1650), in just 20 min using supercritical fluid extraction with CO_2 at two different pressures. At 5000 psi non polar compounds and low molecular weight PAHs are removed, and a 8000 psi extraction yields a cleaner extract containing mainly polar compounds. The use of electrochemical detector for quantification of 1-NP further enhances the selectivity of the overall method sonce it responds only to the electrochemically reducible analytes. Hence by employing a SCFE for sample preparation followed by electrochemical reduction the multi-step, time consuming, lengthy and rather difficult separation schemes can be avoided. The whole process of Soxhlet extraction and fractionation as practiced presently in environmental laboratories would take at least 48 hours of sample handling before the extract could be analyzed. The quantitative results are in excellent agreement with the NIST certified value. GC-MS was used for positive identification of the 1-NP peak.

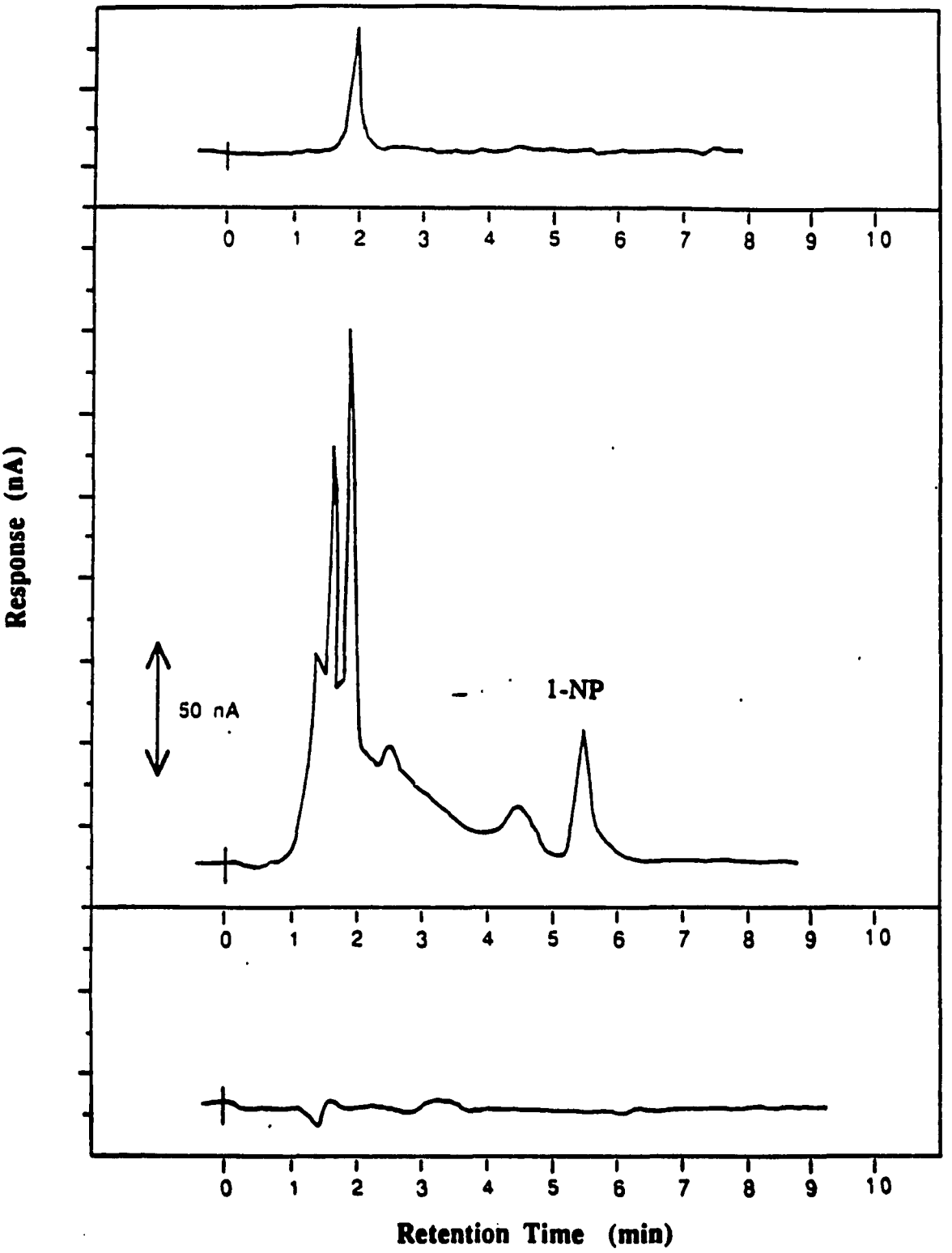

Figure 7. Chromatograms of various supercritical-fluid extracts obtained by changing the pressure used in the extraction or the potential used in the detection. A. HPLC/ECD of 8000 psi extract of two-step SCFE of NBS SRM 1650. Ag electrode potential, -0.5 V vs. calomel. B. HPLC/ECD of 8000 psi extract of two-step SCFE. Ag electrode potential, -1.1 V vs. calomel. C. HPLC/ECD of 5000 psi extract of two-step SCFE procedure. Ag electrode potential, -1.1 V vs. calomel.

Chapter 8

ISOLATION AND CHARACTERIZATION OF OXY-POLYNUCLEAR AROMATIC HYDROCARBONS (oxy-PAHs)

INTRODUCTION

Polynuclear aromatic hydrocarbons (PAHs) and their oxygenated derivatives have been a subject of scientific investigation for the last two decades because of their suspected carcinogenic, mutagenic activities (1, 3, 13). Of the many sources of these pollutants in our environment, especially urban, the most important are vehicle emissions, retort fumes, burning of coal, and incineration. There exists an extensive literature on the characterization and quantitation of PAHs but the field of investigation of oxy-PAHs is relatively new and has not been much explored (4-16). Their isolation and characterization poses a challenge to the analytical chemists because their usual concentrations are very low (8-10 μg/1000 cu. m. of urban air (1).

Recently there has been some work on identification and quantification of fractions of diesel aerosol extracts containing oxy-PAHs. In all these methods workers have adopted the Soxhlet extraction method, either with pure solvents such as dichloromethane or toluene, or a mixture of these solvents. This method is extremely time consuming (8-24 hours) (4-16). The Soxhlet extract is fractionated using various techniques such as HPLC (4, 12, 16), column fractionation (5, 7, 11, 13), or solid phase extraction on silica cartridges (17). A purer oxy-PAH fraction is obtained if solid phase extraction is followed by chromatography on Sephadex LH 20 (18). The different fractions obtained have been analyzed using GC-MS. Although supercritical carbon dioxide has been successfully employed either as single component or with some entrainers for the supercritical chromatographic study of various oxy-PAHs (8-10), there is no literature describing its use in quantitative extraction of these compounds.

The multiple sample preparation methods suffer from low recoveries and poor precision, the two most important factors most desired in trace level isolation determination methods. On the other hand supercritical fluid extraction presents a viable alternative sample

preparation technique compared to the time consuming, inefficient and labor intensive methods currently in practice. In this chapter we report the evaluation of parameters governing the efficiency of extraction of the selected oxy-PAHs and a method of simultaneous isolation and fractionation of a class of polar compounds that could be used for analyses of complex matrices like diesel exhaust particulate.

EXPERIMENTAL

Chemicals

p-Benzoquinone, 1,4-naphthoquinone, anthraquinone, 9,10-phenanthracenequinone, 5,12-naphthacenequinone, benz[a]anthracene-7-12-dione, and acenaphthoquinone were obtained from Aldrich and used without further purification.

Preparation of synthetic mixture of Standards : A synthetic mixture of the standards was prepared by dissolving approximately 10 mg, accurately weighed quantities of each compound in distilled Anal R grade dichloromethane and diluted to 100.00 mL.

Apparatus

Supercritical fluid extractor

A system capable of withstanding high pressure was used to perform supercritical fluid extractions, the plumbing and procedural details of which have been already described (Chapter 2).

HPLC

HPLC analysis was accomplished with a lab-constructed HPLC with a thin layer reductive mode electrochemical detector (Appendix 1). A mobile phase composed of

acetonitrile-water containing 0.05M sodium perchlorate (65/35,V/V) provided adequate separation with a short run-time. Based on previous studies, an applied potential of -1.2 V versus SCE offers excellent sensitivity for the 7 oxy-PAHs. The identity of the peaks was confirmed by comparing the LC retention times with those of authentic samples by co-chromatography.

GC

The GC (HP 5880) used was equipped with an FID; a 30 meter, DB-5 bonded phase, fused silica open tubular column; and split injection (split ratio 1:100). Helium was used both as carrier and auxiliary gas. The column was kept initially at 100 ^{0}C for 2 min. and temperature programmed 5 ^{0}C / min. to the final column temperature, 220 ^{0}C, which was held for a period of 3 min. The temperature of the injection port and detector were maintained at 325 ^{0}C. Under these conditions all compounds gave well separated peaks. Quantitation was done by comparing the automatically computed peak areas obtained by injecting the same volumes of extracted and unextracted samples of standard mixture.

PROCEDURE OF EXTRACTION

To perform SCF extraction, the extraction chamber was disassembled and cleaned with acetone and cyclohexane and dried. A known volume (usually 0.5 mL or 1 mL) of synthetic mixture was streaked onto a glass fiber filter paper (dia. 5.5 cm.) and was placed into the extraction chamber. Glass wool was placed into the ends of the extraction chamber. The extraction chamber was reassembled. With all valves open, carbon dioxide under very low pressure flowed through the extraction chamber and vented into the atmosphere, to remove any air present in the system. The valves were then closed. The system before the extraction chamber was pressurized with carbon dioxide until the desired pressure was obtained. The valve prior to the extraction chamber was opened fully and the

entire system was pressurized. After a period of 20 min the valve after the extraction chamber was carefully partially opened, and the supercritical carbon dioxide containing the extract passed through the second valve and depressurized. The extract was collected on a trap containing silica gel. The silica gel trap was eluted with 10 ml of dichloromethane/acetone mixture (1:1, v/v). The solvent mixture was evaporated to dryness under vacuum using a rotary evaporator and reconstituted either in 1 mL dichloromethane for GC or in 1 mL mobile phase for HPLC.

RESULTS AND DISCUSSION

Extraction efficiency

To determine the optimum conditions for quantitative extraction of the selected oxy-PAH, the effect of pressure and time of equilibration was studied at two different temperatures. Each sample was extracted in triplicate for 20 min with carbon dioxide over the pressure range of 5000 to 8000 psi at 45 and 60 ^{0}C. The influence of pressure on % extraction is indicated in Tables 1 and 2 for 45 and 60 ^{0}C, respectively. It is evident that at both temperatures the extraction efficiency increases with increase in pressure. For 6 of the 7 oxy-PAHs examined, a plateau in recovery was reached near 8000 psi. This is attributed to an increase in the solubility of these quinones in carbon dioxide as its density increases under diffusion-controlled conditions. Further, below 5000 psi no appreciable extraction of these compounds was observed. To study the effect of equilibration time on extraction efficiency, the extractions were performed at 8000 psi and 60 ^{0}C for times varying from 5 min to 20 min. As shown in Table 3, the extraction for each of these compounds was essentially complete during the first 15 min using pure carbon dioxide.

Table 1: Effect of Pressure on extraction efficiency# of oxy-PAHs*

Compound	Pressure (psi)			
	8000	7000	6000	5000 (psi)
1,4-Naphthoquinone	91	85	60	51
Benzoquinone	87	83	67	56
9-Fluorenone	86	76	70	55
Acenaphthequinone	80	70	45	31
Anthraquinone	96	92	67	54
Phenanthracenequinone	74	71	43	27
7H-Benz {de}anthracene-7-one	92	90	72	46
5,12-Naphthacenequinone	77	62	42	19

***Temperature of extraction = 60 ^{0}C**
Time of Equilibration = 20 min.
 # percentage recovery

Table 2. Effect of pressure on extraction efficiency[#] of oxy-PAHs

Compound	8000	7000	6000	5000 (psi)
1,4-Naphthoquinone	65	52	44	39
Benzoquinone	69	57	48	41
9-Fluorenone	64	56	32	29
Acenaphthequinone	55	38	23	14
Anthraquinone	84	77	68	56
Phenanthracenequinone	44	32	26	11
7H-Benz {de}anthracene-7-one	80	73	65	49
5,12-Naphthacenequinone	42	28	22	16

*
Temperature of extraction = 45 °C
Time of Equilibration = 20 min.
 # percentage recovery

Table 3. Effect of equilibration time on extraction efficiencies#

Compound	5 min	10 min	15 min	20 min
1,4-Naphthoquinone	50	71	88	90
Benzoquinone	57	72	80	85
9-Fluorenone	55	63	76	82
Acenaphthequinone	59	72	80	84
Anthraquinone	60	74	90	94
Phenanthracenequinone	43	51	69	70
7H-Benz {de}anthracene-7-one	62	74	80	89
5,12-Naphthacenequinone	38	53	68	74

Temp. of extraction = 60 °C
Pressure of extraction = 8000 psi

 # percentage recovery

Additional extractions were performed to determine the effect of a polar entrainer such as methanol on recovery of these polar compounds using pure carbon dioxide and carbon dioxide modified with 5% by volume of methanol as extractants. A calculated volume of methanol was added directly to the sample matrix in the extraction chamber. These extractions were performed at 8000 psi and 60 ^{0}C. Extracts were collected at 5 min intervals and analyzed as described in the experimental section. As shown in Table 4 for incomplete extractions, enhanced extraction rates can be obtained through the direct addition of 5% (v/v) of methanol to the matrix prior to extraction.

Evaluation of the collector efficiency

The efficiency of the collection device is crucial to the overall success of the isolation procedure, i.e. whatever is extracted should be retained on the collector. The performance of a packed guard column as a collection device was evaluated by performing several extractions in which different packing materials were utilized for otherwise identical extractions of 100 μg of anthraquinone from chromatographic paper. The recovery results are shown in Table 5. It is clear that the recoveries and reproducibilities are independent of packing material and are satisfactory for quantitative work.

For comparison, a similar extraction was conducted and the extract trapped on a cold trap , constructed from a crimped stainless steel hollow U tube (1/16 in. x 0.005 in ID) immersed in a liquid nitrogen bath. Chromatographic analysis of the trap wash yielded an efficiency of only 55%. Perhaps the efficiency of this simple trap would be improved by packing the hollow tube with stainless steel balls to provide extra surface area to the trap.

Finally, the efficiency of the packed trap was evaluated by passing the exiting carbon dioxide through a downstream liquid trap. For this purpose, an 8 in. length of 1/16 in O.D. x 0.010 in. stainless steel tubing, connected in series with the guard column trap, packed with silica was inserted into a 250 ml volumetric flask containing 100 ml of methylene chloride. Following depressurization, the connecting tubing was rinsed with methylene chloride and combined with the liquid in the trap. Analysis of the liquid trap showed that no breakthrough of the oxy-PAHs had occurred.

Table 4. Effect of Entrainer on the extraction efficiencies of oxy-PAHs

Compound	Extraction Efficiency (%)	
	CO_2	CO_2+ CH_3OH (5%, v/v)
1,4-Naphthoquinone	50	75
Benzoquinone	57	77
9-Fluorenone	55	68
Acenaphthequinone	59	72
Anthraquinone	60	84
Phenanthracenequinone	43	67
7H-Benz {de}anthracene-7-one	62	81
5,12-Naphthacenequinone	38	57

Temp. of extraction = 60 °C
Pressure of extraction = 8000 psi
Time of equilibration = 5 min

Table 5. Evaluation of Packed Trap as Collector.

Packing Material	Percentage Recovery	RSD (n=3)
Silica	92	3.9
Alumina	87	3.3
Celite	89	2.7
C_{18}	92	1.6

Selectivity

The potential of supercritical fluid extraction to provide class-selective fractionation was investigated by sequentially extracting a sample composed of 200 μg each of pyrene, phenanthrene, benzoquinone, 1,4 naphthoquinone and anthraquinone at low pressure and then at higher pressures. The model sample was initially extracted with carbon dioxide at 3500 psi and 60 ^{0}C for 5 min. The trap was removed and reserved for analysis by capillary GC/FID. Later, the sample was re-extracted for an additional 15 min. at 8000 psi and 60 ^{0}C. This extract was collected and analyzed using the LCEC methodology described in the experimental section. The chromatographic results for the low and high pressure fractions are shown in Tables 6 and 7, respectively. Approximately 82 % of the PAH's was recovered in the first fraction while greater than 95% of the oxy-PAHs was retained on the paper substrate. These compounds were quantitatively isolated in the second fraction after the pressure was raised to 8000 psi. Figure 1 shows the LCEC chromatogram of the high pressure extract. The absence of interfering components is evident, although some overlap of components occurred in the two fractions. Since most of the environmentally important PAHs occurring in air filter samples can be quantitatively and selectively extracted at low pressures (3500-4000 psi) (19), leaving the polar compounds in the high pressure fraction, the simultaneous extraction-fractionation using supercritical carbon dioxide in such cases seems feasible.

Minimum extractable concentration levels for various oxy-PAHs

Extraction limits for various quinones were estimated by using a challenging matrix, such as carbon black (Darco, G-60, Fischer Scientific Co, Pittsburgh, PA) to mimic a diesel exhaust sample as close as possible . Several carbon black samples were spiked with progressively lower amounts of the 7 oxy-PAHs and extracted with carbon dioxide for 15 min at 8000 psi and 60 ^{0}C. The extracts were analyzed using LCEC, and the extraction limit for each component was estimated as the concentration level above which the recovery dropped below 75%. These limits are summarized in Table 8. These limits suggest that SCFE can be successfully applied to the isolation of these analytes at trace level concentration in complex matrices.

Table 6. Composition of low pressure extract*

Component	Concentration, μg/g	Percentage recovery
Pyrene	166	83
Phenanthrene	162	81
Benzoquinone	10	5
1,4-Naphthoquinone	4	2
Anthraquinone	0	0
5,12-Naphthacenequinone	0	0

* 3500 psi, 60 ^{0}C, 5 min.

Table 7. Composition of high pressure extract*

Component	Concentration, μg/g	Percentage recovery
Pyrene	28	14
Phenanthrene	24	12
Benzoquinone	176	88
1,4-Naphthoquinone	184	92
Anthraquinone	186	93
5,12-Naphthacenequinone	150	75

* 8000 psi, 60 ^{0}C, 15 min.

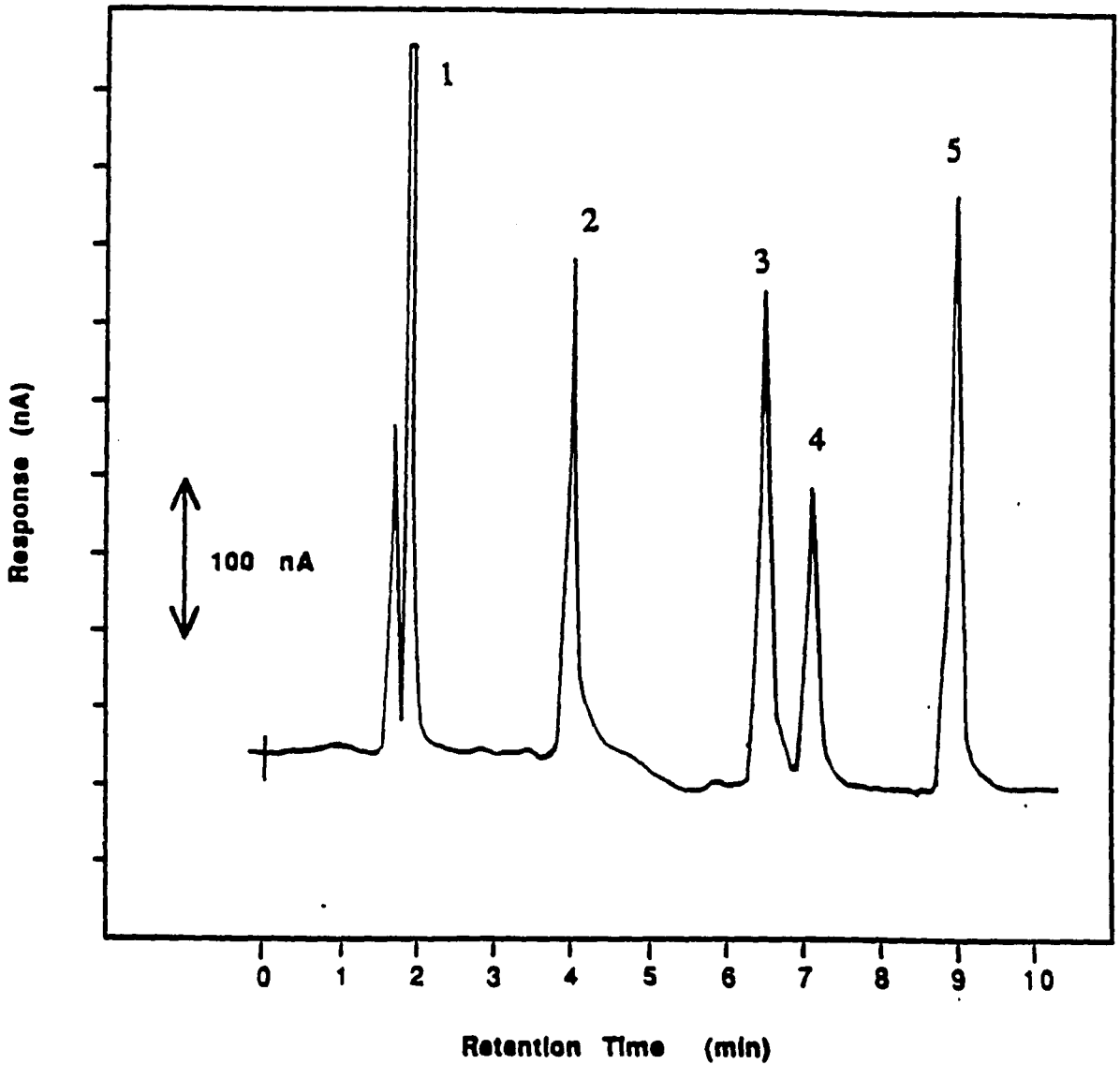

Figure 1. Chromatogram of a series of quinones. Chromatographic conditions: column, 150 x 4.6 mm ID Alltech C-8 (10 μm); mobile phase, acetonitrile-aq. 0.05 M sodium perchlorate (65+35, v/v); flow rate, 1.5 mL/min; applied potential, -1.2 V *vs* SCE. Peak identities: 1, benzoquinone (83.6 ng); 2, 1,4- naphthoquinone (320 ng); 3, anthraquinone (260 ng); 4, phenanthracenequinone (76 ng); 5, 5,12-napthacenequinone (233 ng).

Table 8. Lowest extractable concentrations for various oxy-PAHs*

Compound	Extraction Limit (μg/g)
1,4-Naphthoquinone	1
Benzoquinone	5
Acenaphthequinone	15
Anthraquinone	3
Phenanthracenequinone	20
7H-Benz {de}anthracene-7-one	10
5,12-Naphthacenequinone	25

* 8000 psi, 60 ^{0}C, 15 min.

Precision

The ability of SCFE followed by HPLC with electrochemical detection procedures to yield reproducible results was investigated by extracting and analyzing replicate carbon black samples containing 10 μg/g of 1,4 naphthoquinone. The coefficient of variation for 5 replicate extractions and subsequent determinations ranged from 2.3 to 4.9 % for within-day work and from 3.4 to 7.6 % for between-day studies.

Chapter 9

Isolation of Parathion Residues from high lipid food matrices

INTRODUCTION

Parathion belongs to an important class of organo-phosphorous pesticides used on cash crops such as cotton seed, ground nut, soybean, and a number of legumes and cereals (1, 2, 3). Parathion is rather unstable; it decomposes with time to a number of hazardous chemicals such as nitro phenol. Hence it is important to analyze treated crops for the trace amounts of parathion and its degradation products (1-5). The isolation of parathion from high lipidic matrices is complicated by the fact that along with analyte, a large amount of fat and other substances are extracted. The co-extractants not only make the extraction procedure difficult but also make direct GC or HPLC analysis of the extract almost impossible, since the underivatized triglycerides can clog the GC and HPLC columns and their detectors . The thermal lability of parathion and its hydrolysis to nitro phenol add to the problems encountered in its isolation and characterization (1, 3).

The pace of development in the field of isolation of organophosphorous pesticides has been rather slow, as it is evident by the limited number of research publications available (2). Current methodologies to isolate parathion include a multiple step solvent extraction of the matrix followed by liquid-liquid partitioning to remove the lipids and other undesirable co-extractants. The methods recommended by the Association of Official Analytical Chemists (AOAC) and the Food and Drug Administration's (FDA) Pesticide Analytical Manaual (PAM) require a sequential extraction of the ground sample matrix first with petroleum ether and then with diethyl ether and petroleum ether (50/50). The method, though slow and labor intensive, is still popular because of the simplicity of the procedure and its capability of handling large number of samples. However, the multi-step process and formation of emulsions can lead to serious sample losses. Even after multi-step extraction, a fat clean-up step is invariably involved before the extract can be analyzed by any analytical technique (1, 2).

The most popular method of fat removal in the past has been the liquid-liquid partitioning of the extract with immiscible solvent pairs such as hexane-acetonitrile (1). Though simple, the process is tedious, labor intensive, has poor precision, and is not amenable to automation. Other widely used methods include gel permeation chromatography (6-9), solubilization in sulfuric acid (10), metal precipitation (11), sweep-codistillation (12), and solid-phase extraction (13-16). Of all the methods listed here, solid-phase extraction (SPE) has gained popularity in recent times because

of its ease of operation, fast speed, lack of emulsion formation, commercial availability of disposable, ready-to-use cartridges, and amenability to automation. Nevertheless, the technique suffers from serious drawbacks such as large sample volume requirements, which may hamper the quantitative transfer of extracts to the cartridges. The sample volumes in the case of viscous extracts may sometimes exceed the break-through volume of the analyte, thus resulting in loss of selectivity and poor recoveries.

Even though the high temperatures employed in GC can result in thermal degradation of parathion, use of GC has been frequently reported for the quantitation of this analyte despite poor reproducibilities (17). Since HPLC procedures do not usually involve high temperatures, the danger of thermal degradation is eliminated. However, the most commonly used UV detectors do not offer the high sensitivity and selectivity necessary for trace level analysis (18-20). On the other hand, the electrochemical detector offers excellent sensitivity and high selectivity, since it responds only to the electroactive species in the extract. The details of design, operation, and applications of electrochemical detectors are available in the literature (21-23). Kohen and Huber were the first to detect parathion amperometrically from contaminated lettuce extracts. The results were rather poor with high detection limits and poor precision because of dissolved oxygen and electrode clogging (24). Ding and Krull attempted a quantitative photolysis of parathion with UV radiation followed by oxidative amperometry of the product to enhance selectivity and detection limits (25). Clark and Goodin used a glassy carbon electrode in the reductive mode in conjunction with a UV detector and reported detection of parathion in a number of contaminated green vegetables (26).

Based on our successful single step isolation of analytes such as menadione (Chapter 4) and vitamin K_1 (Chapter 5) from lipid-rich matrices, animal feed and infant formulas, respectively, supercritical fluid extraction of parathion from various oil-laden matrices was investigated. Various parameters governing the SCFE, HPLC separation and electrochemical detection were studied to develop a quantitative, trace level, high precision, sensitive, selective and accurate method for isolation and quantitation of parathion from various oil-rich matrices.

EXPERIMENTAL

Chemicals

Methyl parathion and ethyl parathion of Anal R grade were obtained from Chem Services. Nitrophenol was obtained from Aldrich. HPLC-grade methanol, acetonitrile, and hexane were purchased from J. T. Baker. Buffer salts were acquired from Fisher and used without further purification. In-house glass distilled water was used in all studies.

Apparatus

The design and construction of the supercritical fluid extractor incorporating a solid-phase collector has been thoroughly detailed in Chapter 2. The syringe pump based LCEC system employing a silver working electrode has been described in Appendix 1.

Preparation of standards

Stock solutions of parathions were prepared by transferring 125 mg, accurately weighed, to 100 mL volumetric flasks and diluting to the mark with acetonitrile. Working solutions ranging from 12.5 μg/mL to 12.5 ng/mL were prepared by diluting the stock solutions with acetonitrile or methanol.

Extraction

Cereals, legumes, and oil seeds were obtained from local supermarkets. The weighed samples were ground with sufficient glass bead powder to form a homogeneous, coarse powder. The ground samples were spiked with 1 mL of the pesticide in methanol and the solvent was allowed to evaporate at room temperature. The sample was transferred to the extraction chamber and extracted for 15 min, at 8000 psi and 65 ^{0}C. The back-pressure regulator was carefully cracked open allowing the crude extract to be uniformly deposited onto the sorbent collector. Subsequently, the cartridge, valve and connecting fittings were backflushed with 2 mL of acetonitrile. The eluate was evaporated in a rotary evaporator to dryness, reconstituted in 1 mL of mobile phase and then subjected to HPLC analysis.

HPLC determination

After the acetonitrile and aqueous buffer were mixed in the proper proportions, the mobile phase was degassed with nitrogen for 15 min to ensure removal of oxygen. The mobile phase was loaded into the syringe pump cylinder without exposure to air by proper positioning of the valves on the liquid chromatograph manifold. Following equilibration of the baseline at a flow rate of 2 mL/min., 1 mL aliquots of the sample were gently bubbled with mobile phase-saturated nitrogen for one min, and 20 µL introduced into the HPLC using the chromatographic apparatus. The parathion peak was identified on the basis of retention time and quantitated by comparison with a calibration curve.

RESULTS AND DISCUSSION

Electrochemical characteristics of parathion

An HDV was generated by repeated injections of a mixture containing 12.5 µg/mL each of ethyl and methyl parathion at 18 applied potentials ranging from -0.2 to -1.0V with respect to SCE (Fig. 1). On the basis of these measurements, an applied potential of -0.85V was selected for both methyl and ethyl parathion as the minimum applied potential at which the current reaches the limiting current plateau. The electrochemical response to p-nitrophenol, a major degradation product of methyl- and ethylparathion was briefly examined and was found to reach only 75% of its maximum sensitivity under the chosen conditions.

Effect of pH

The dependence of the signal-to-noise ratio on pH is illustrated in Figure 2. It may be concluded from this figure that the optimum S/N ratio is achieved with buffers of pH 5-7. At extremely high pH levels there is a danger of parathion being hydrolysed to nitrophenol and at extreme low pH levels the detector noise increases because of increased hydrogen evolution.

Detector response

A series of parathion solutions in acetonitrile covering the concentration range of 12.5 ppb to 125 ppm was prepared to study the detector response. For each concentration 3 replicate injections were made and log-log plots of average peak-height vs. concentration were constructed. The calibration curve for ethyl parathion, illustrated in Figure 3, exhibits a linear dynamic range of at least 3 orders of magnitude.

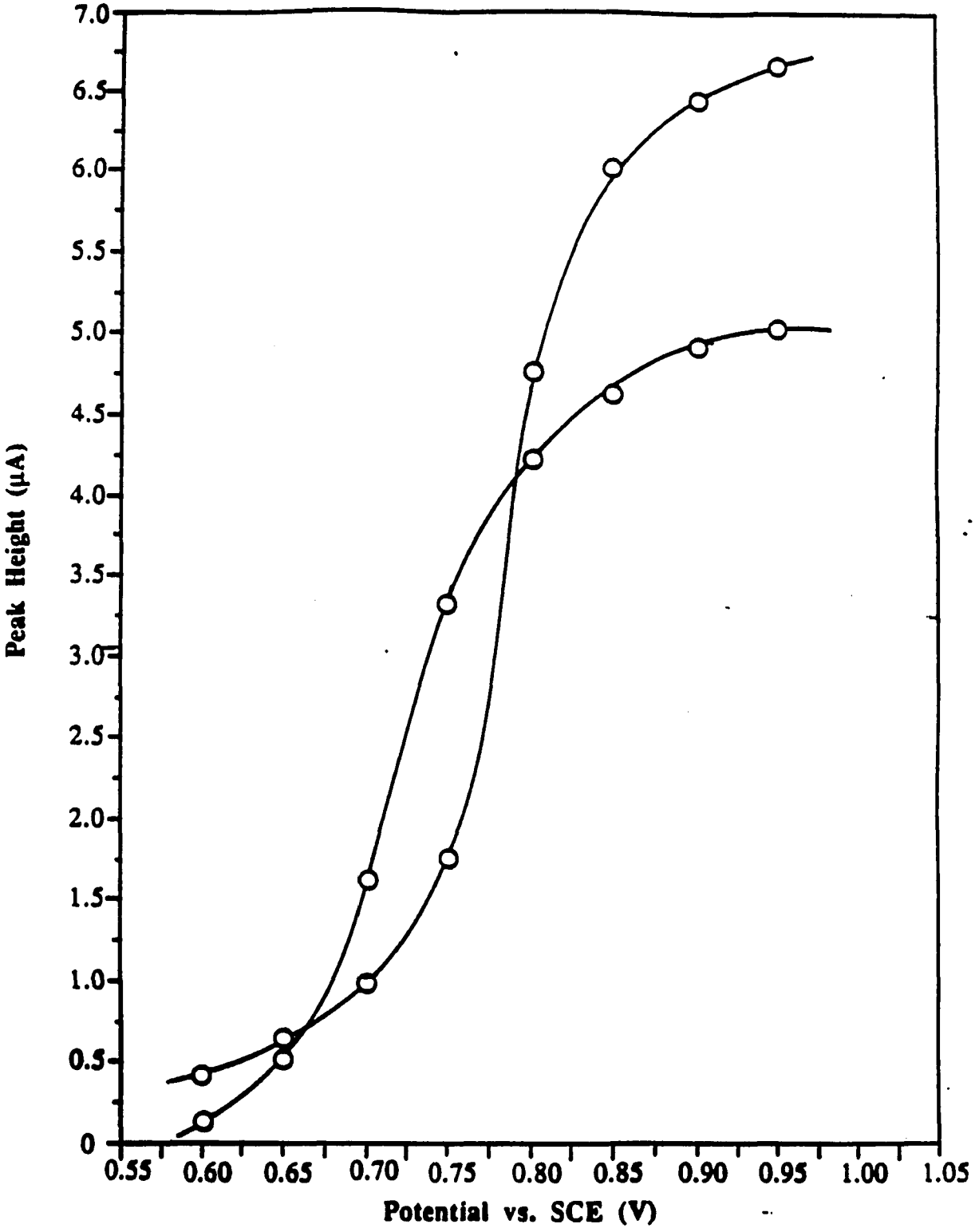

Figure 1. Hydrodynamic voltammogram of methyl and ethyl parathion on silver cathode *vs* SCE. Column: 150 x 3.9 I.D. mm µ-Bondapak C-18 (10 µm). Mobile phase: Acetonitrile -.025 M aq phosphate buffer, pH 6.8 (50+50, v/v). Sample size: 20 µg per 20 µL mobile phase of each parathion injected at each potential setting.

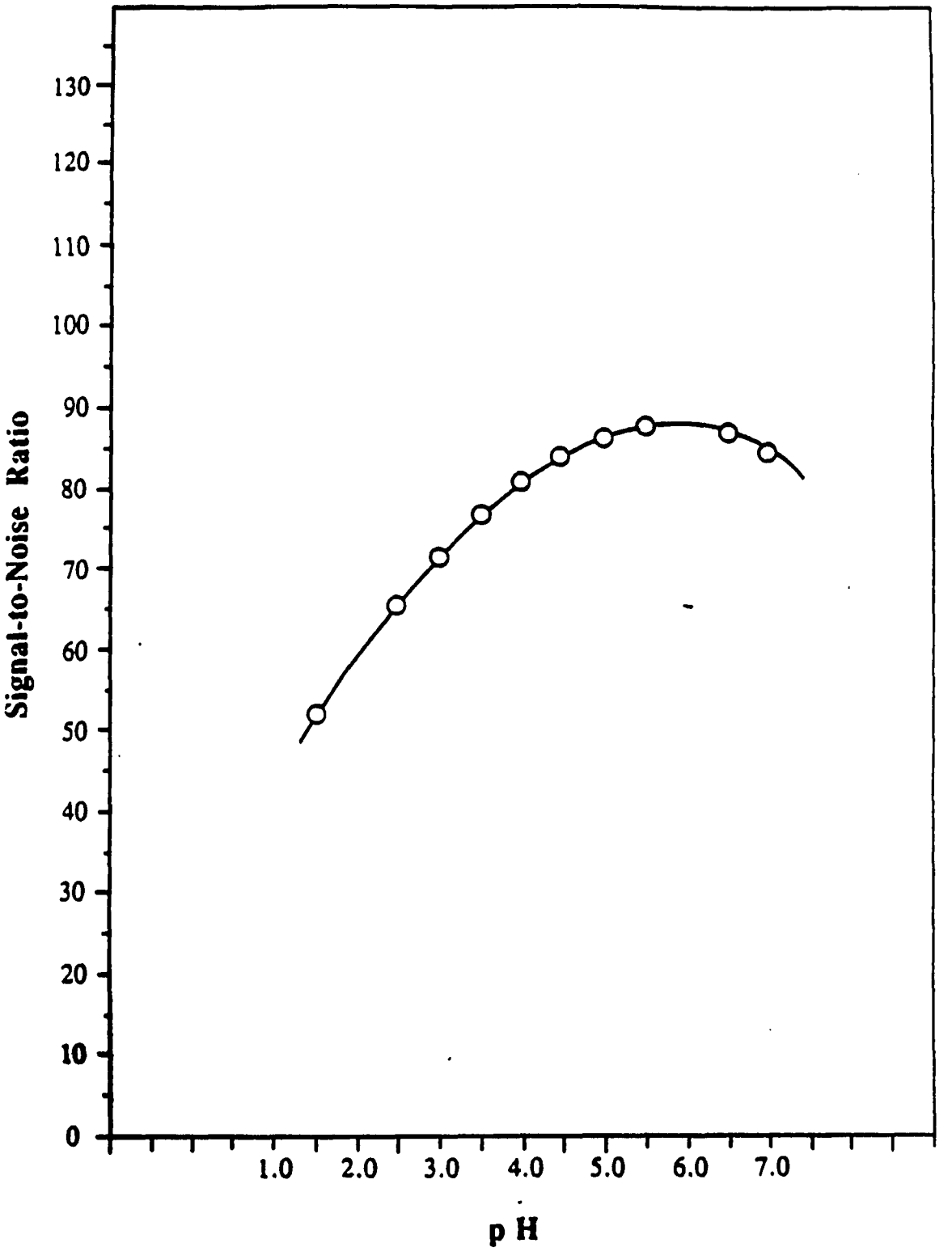

Figure 2. Influence of pH on signal-to-noise ratio for methyl parathion. E applied, -0.85 vs SCE; other chromatographic parameters as in Figure 1.

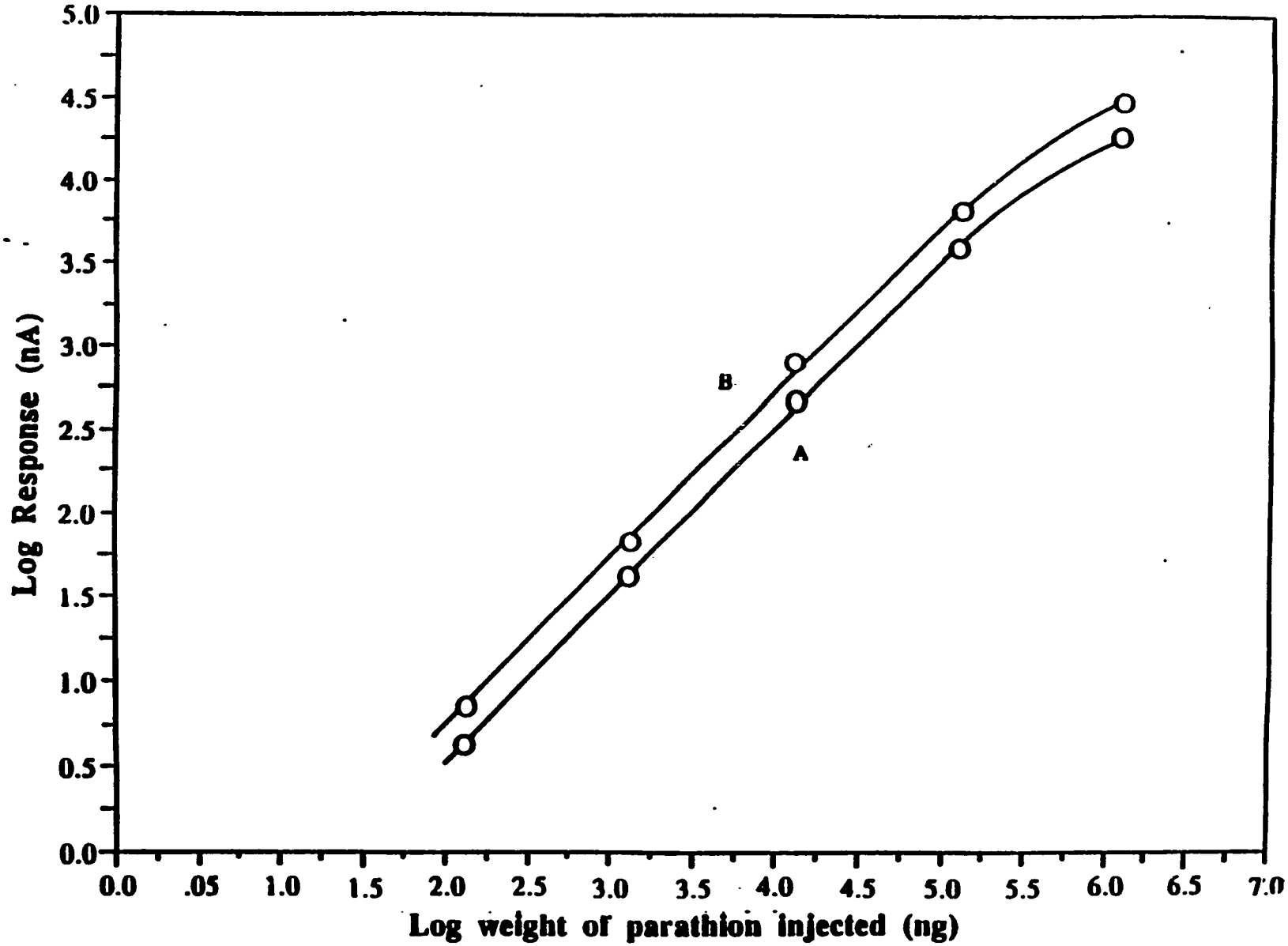

Figure 3. Linearity of detector response to amounts of (A) methyl and (B) ethyl parathion in 20 µL samples. Column, 150 x 3.9 I.D. mm µ-Bondapak C-18. Eluent, acetonitrile-aq phosphate buffer, pH 6.8 (50+50, v/v). Flow rate, 2.0 mL/min. Potential of working electrode, -1.2 V *vs.* SCE.

The equations of the best-fit line computed using linear regression analysis for ethyl and methyl parathion were:

$$y\ (nA) = 2.8\ (ng) + .009 \quad \text{(ethyl parathion)}$$

and

$$y\ (nA) = 2.0\ (ng) + .015 \quad \text{(methyl parathion)}$$

Even though, the electrochemical reaction is the same in both parathions, the slight differenece in their slopes is probably due to the differences in purities of the the samples used here. The lower limit of detection for both parathions was approximately 200 ng per 20 µL injection, allowing for a signal to noise ratio of 3 to 1. The measured peak heights for repetitive injection of the extract were highly reproducible, with an RSD of 1.5%.

Chromatography

Separation of methyl and ethyl parathion from interfering degradation products was achieved by reversed-phase HPLC on a µ-Bondapak RP C_{18} column with an acetonitrile-aqueous phosphate mobile phase. In initial studies, capacity factors (k') for ethyl parathion, methyl parathion, and nitrophenol were measured at different mobile phase compositions, thereby allowing the selection of an optimal mobile phase composition. The influence of acetonitrile content on retention time is shown in figure 4. An acetonitrile content of 50% offers a good compromise between run-time and selectivity. The pH of the phosphate buffer used in the mobile phase was crucial to the peak symmetry of the three components. In particular, the effect of pH on the peak shape of nitrophenol is dramatic. Tailing and a decrease in capacity factor was observed when the pH exceeded 7.5 because of partial ionization at higher pH levels. In some cases, even multiple peaks were obtained for a standard solution of nitrophenol chromatographed with such buffers. Considering this problem and the pH stability of the column packing, the pH was maintained at 6.8 for all studies.

Figure 5 is a typical electrochemical chromatogram for a synthetic mixture of methyl parathion, ethyl parathion, and nitrophenol obtained with a mobile phase consisting of acetonitrile-0.025 M phosphate buffer, pH 6.8, (50/50, v/v) and flow rate of 2 mL/min. The peaks for the parathions were sharp and well separated from each other and from nitrophenol such that the retention times for nitrophenol, methyl parathion, and ethyl parathion were 2.0, 3.8, and 5.0 min., respectively. The total run time was only 7 min.

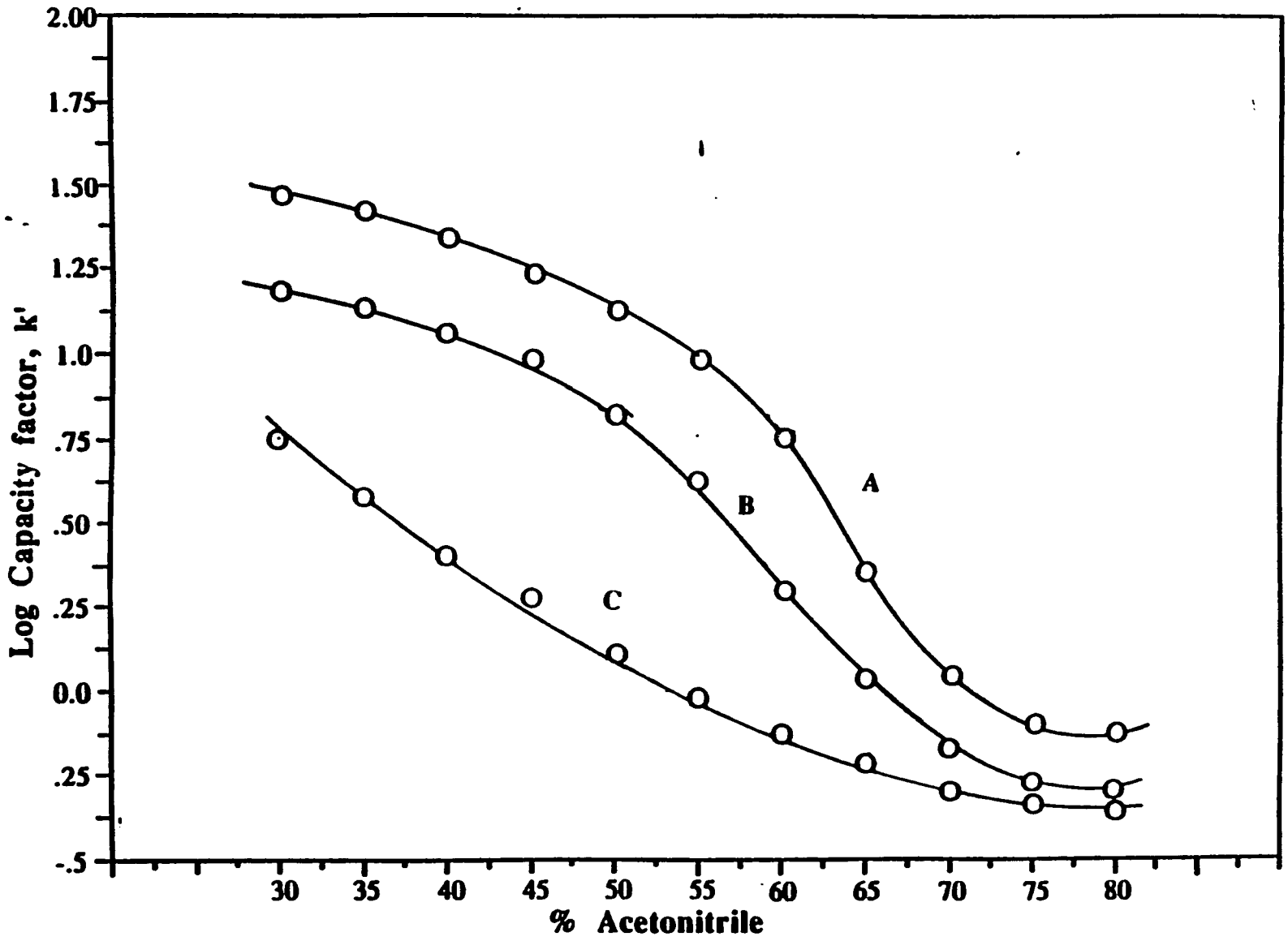

Figure 4. Dependency of capacity factor of (A) methyl parathion, (B) ethyl parathion and (C) nitrophenol on acetonitrile content of mobile phase. Column: 150 x 3.9 mm I.D. μ-Bondapak C-18 (10 μm). Flow rate: 2.0 mL/min. Silver electrode potential: -0.85 V *vs* SCE.

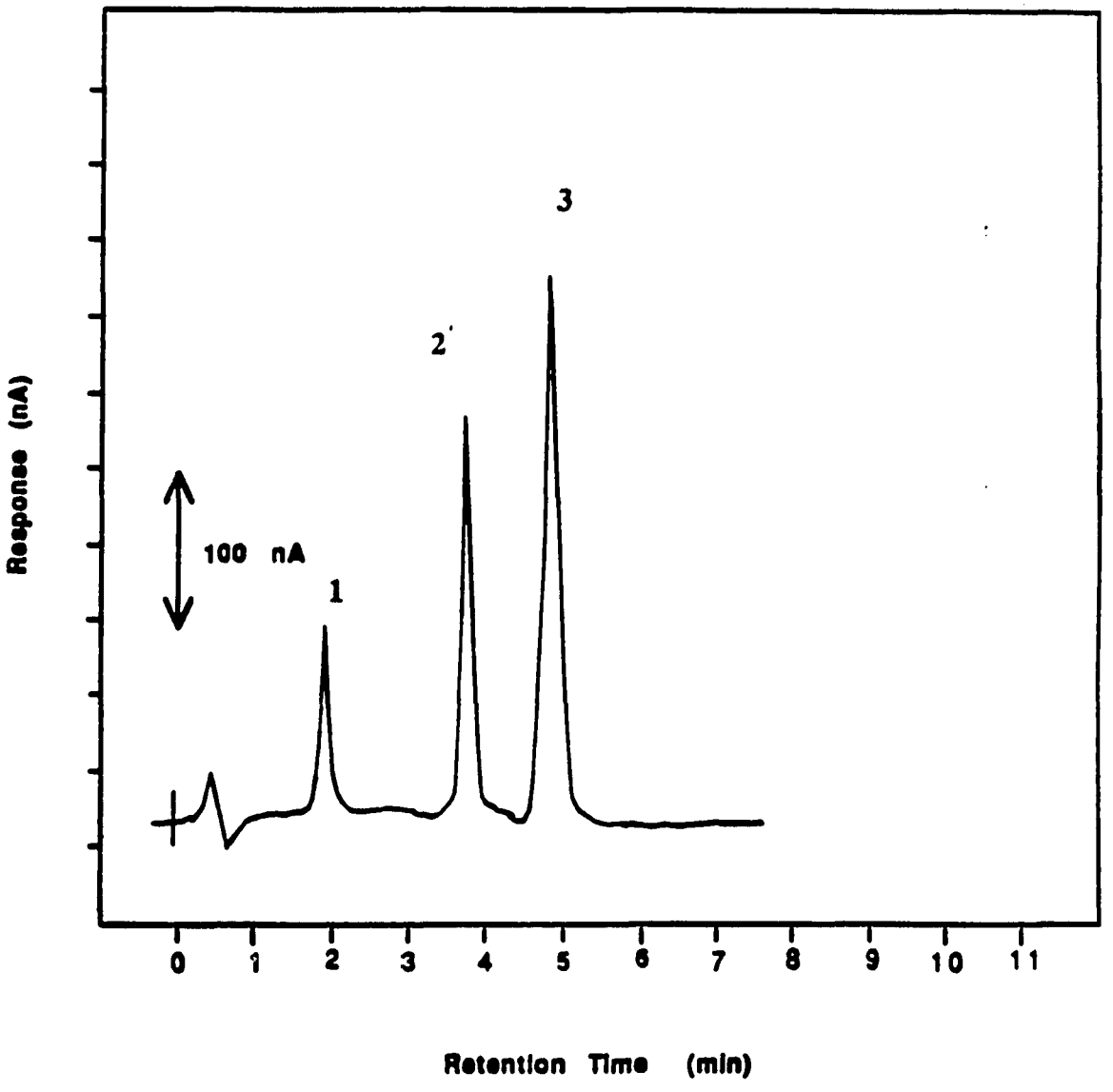

Figure 5. Chromatogram of the separation of ethyl parathion, methyl parathion and nitrophenol on a μ-Bondapak C-18 (10 μm) column by isocratic elution: mobile phase, acetonitrile-aq phosphate buffer, pH 6.8 (50+50, v/v); flow rate 2.0 mL/min; detector potential, -0.85 V *vs* SCE; detector sensitivity 100 nA/V. Peak identites as follows: 1, methyl parathion; 2, ethyl parathion; 3, nitrophenol.

Optimization of sample preparation technique

In order to obtain maximum recovery in the shortest time the SFC extraction parameters were optimized with respect to pressure, temperature and equilibration time. To examine this aspect, 1 g Chromosorb W was fortified with 12.5 μg parathion and extracted at 45 ^{0}C and 60 ^{0}C in the pressure range of 5000 to 8000 psi (Figure 6). Each extraction was performed in triplicate for a period of 15 min. As can be seen, the percentage recovery increased with pressure at both temperatures until a limiting value suitable for quantitation was reached near 7000 psi; the effect was more dramatic at 45 ^{0}C. This is not unexpected since the density of supercritical carbon dioxide is greater at lower temperature. In fact at 6000 psi, a reversal in recovery is seen and the recovery obtained beyond 6000 psi at 45 ^{0}C is slightly greater than at 60 ^{0}C. For a single solute, the cross-over point represents the pressure at which there is a change in the temperature dependence of the solubility. Below this pressure, solubility is principally dependent on solvent density and a decrease in temperature leads to an increase in the density and the solubility. Above this pressure, solubility is principally dependent on solute sublimation pressure. Raising the temperature leads to an increase in the sublimation pressure and therefore an increase in solubility. A similar cross-over effect has been observed in case of aldrin and other volatile compounds (27). Considering the thermal lability of the compounds involved, an extraction temperature of 45 ^{0}C and a pressure of 7000 psi was used through the study. Under these conditions, recoveries of standard methyl and ethyl parathion extracted from Chromosorb W averaged 95%. Additional studies were conducted to evaluate the effect of equilibration time. Figure 7 shows the influence of equilibration time on recovery of methyl parathion at constant temperature and pressure of 45 ^{0}C and 7000 psi. As can be seen, an equilibration time of 15 min. is sufficient for quantitative recovery, after which no change is observed up to at least 30 min.

Linearity of over-all procedure

The linearity of the extraction /clean-up step in the range of 125 ng/g to 12.5 μg/g for parathion was confirmed by carrying out the described procedure on fortified rice samples. The lower limit of extraction was 50 ng/g, which in this case approaches the detection limit of the ECD for parathion.

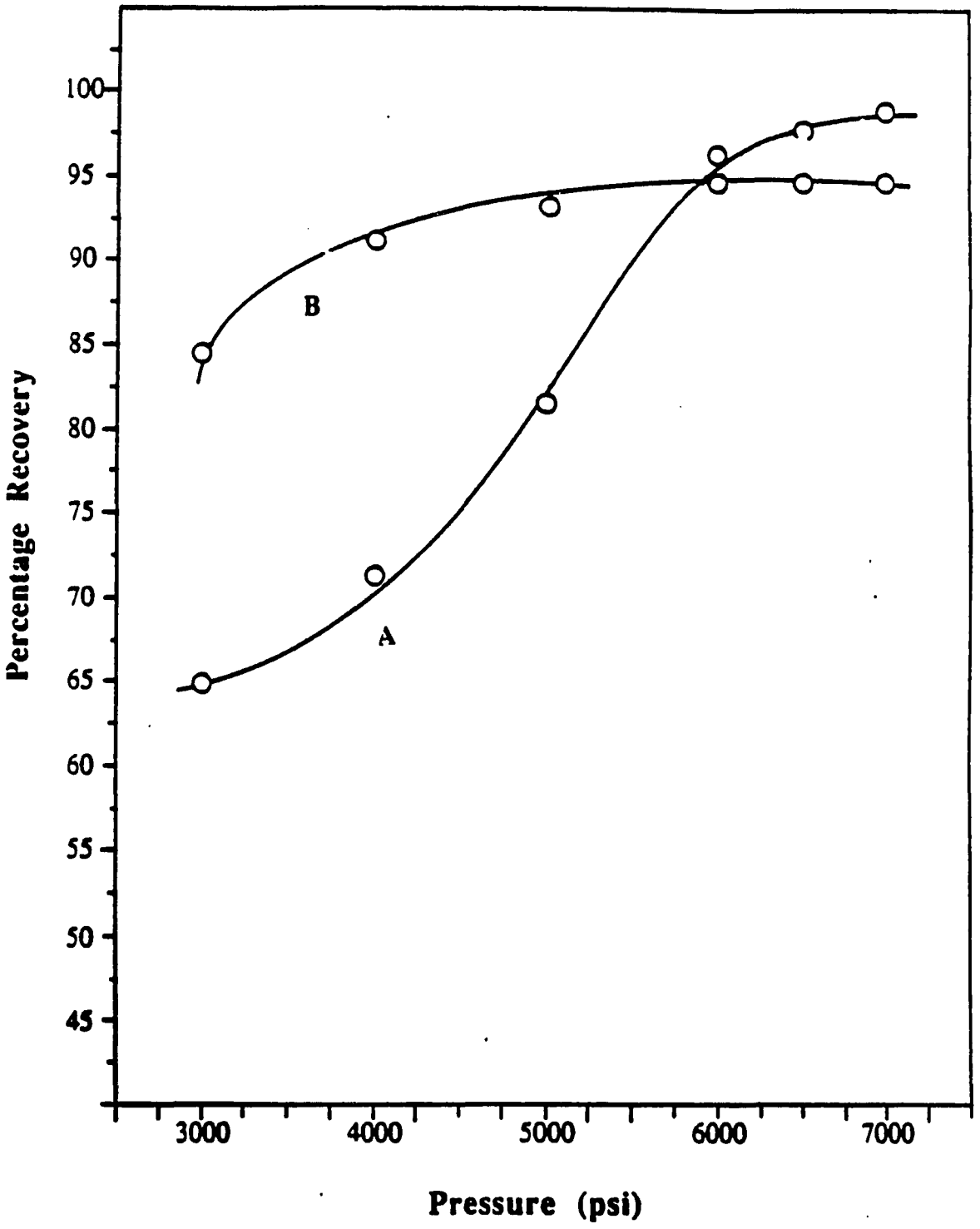

Figure 6. Effect of pressure on efficiency of SFE procedure for ethyl parathion at (A) 60 ^{0}C and (B) 45 ^{0}C. In each case equilibration time was 20 min. A 2 gram cartridge of Chromosorb W fortified with 12.5 μg of ethyl parathion was extracted at each pressure. HPLC analysis conditions as in Figure 5.

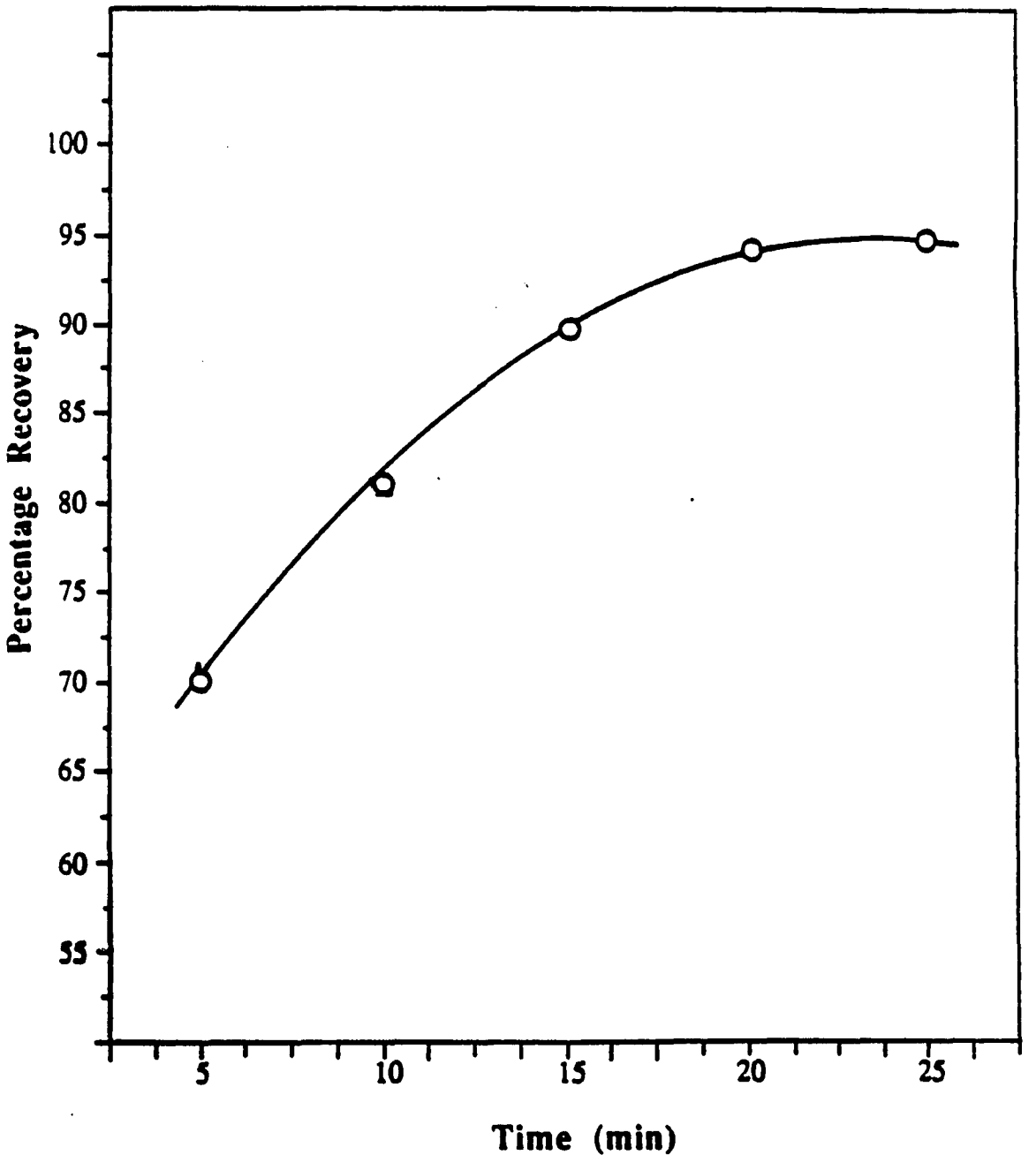

Figure 7. Effect of time on the efficiency of SFE procedure for methyl parathion using 8000 psi CO_2 at 45 °C. HPLC chromatographic analysis conditions as in Figure 5.

Effect of lipid load

To ascertain the interference of lipids on the extraction of parathion, 1 g Chromosorb W fortified with parathion at three different concentration levels was treated with four different amounts of soybean oil, viz. 25, 50, 75, and 100 mg. The samples were then extracted according to the optimum conditions specified above. Table 1 shows the percentage recovery of parathion as a function of initial lipid load. As shown in table 1, recoveries were always 80% or better, approaching complete recovery in most cases, indicating that at these fortification levels the extraction of parathion was not hampered by the presence of relatively high amounts of vegetable fat.

Comparison with Pipet Loading

To examine the advantages of loading solid phase cartridges by SCFE dissolution/precipitation over conventional means, 1 mL of an oily hexane extract containing 100 μg parathion was manually loaded onto a preconditioned cartridge packed with 1.5 g of alumina. The glassware and the implements used to transfer the extract were washed and these rinsings, amounting to 2.0 ml, were also transferred to the cartridge. The excess eluate was collected and the column was further eluted with three 1 mL portions of acetonitrile. Fractions were collected at 0.5 mL intervals in Reacti-vials already containing 0.5 mL of methanol. A plot of response as a function of eluant volume is shown in figure 8. As can be seen, parathion prematurely breaks through at a volume of 2.5 mL, which is equivalent to 1.3 column volumes. Moreover, the collected fractions remained oily in nature, indicating excessive lipid carry-over. The loss of selectivity and efficiency of the SPE cartridge for parathion was attributed to the need for excessive washings with hexane. In contrast to the proposed method, lipid extracts are deposited onto the packing material promoting the efficient partitioning of the pesticide into the acetonitrile eluent. Minimal lipid release also enables extracts to be further concentrated.

Application to Crop Analysis

The efficacy of the SCFE-HPLC-ECD method for isolation and quantification of residual pesticides was tested on parathion-fortified high lipid content corn and peanut seeds. Representative chromatograms of treated peanut and corn samples after supercritical fluid extraction and HPLC with electrochemical detection are shown in figures 9 and 10, respectively. There is excellent separation between methyl parathion, ethyl parathion, and nitrophenol with near baseline resolution. The virtual absence of interferences allows detection limits of less than 50 ng/g to be achieved. The same

Table 1. Recovery of parathion added to the vegetable oil

Oil load (mg)	% Recovery Fortification level of parathion 12.5 (ppm)	1.25 (ppm)	0.125 (ppm)
25	93	87	89
50	85	101	92
75	96	91	84
100	88	80	82

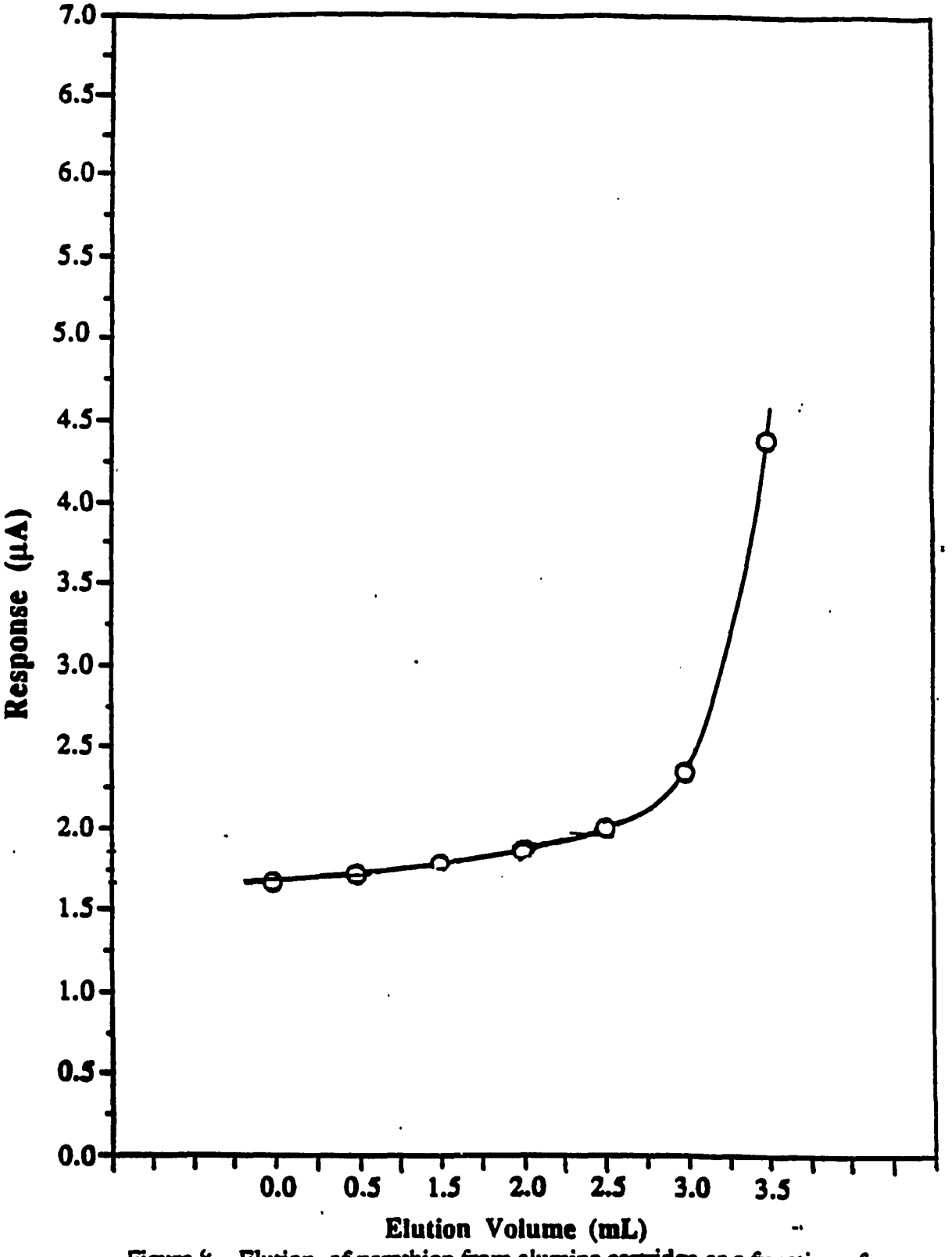

Figure 8. Elution of parathion from alumina cartridge as a function of elution volume. Desorption conditions: eluent, 100 % acetonitrile. Sample size: a 1.5 g of alumina cartridge spiked with 500 μg ethyl parathion per mL of hexane. Chromatographic analysis conditions: column, 150 x 3.9 I.D. mm μ-Bondapak C-18 (10 μm); mobile phase, acetonitrile-aq 0.025 M phosphate buffer, pH 6.8 (50+50, v/v); flow rate, 2.0 mL/min; detector potential, -0.85 V *vs* SCE; current sensitivity, 100 nA/V.

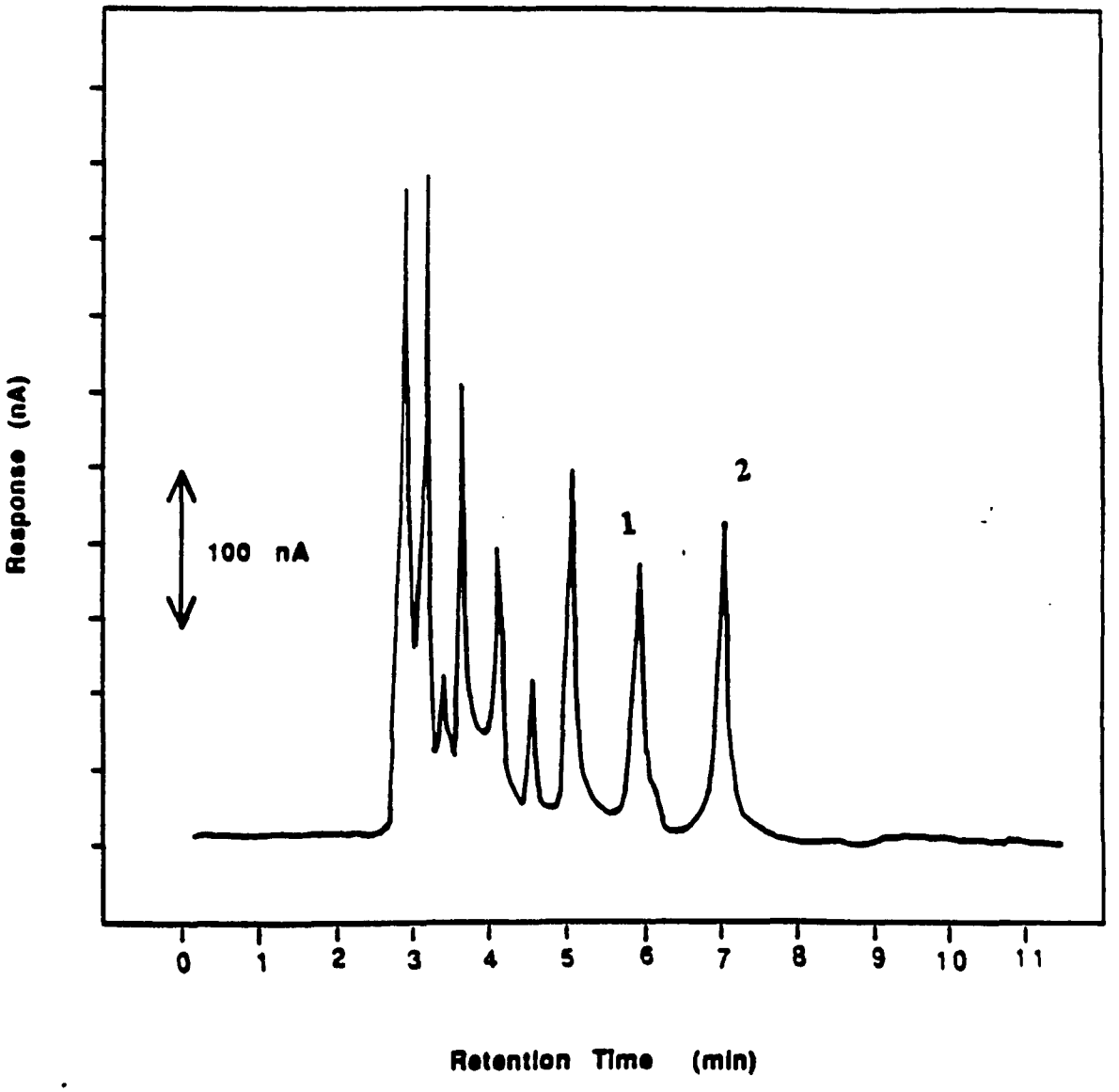

Figure 9. HPLC-ECD chromatogram of a supercritical-fluid extract of a peanut sample treated with methyl parathion (1) and ethyl parathion (2). Extraction conditions: SFE with 8000 psi CO_2 , 45 °C, 20 min. A gram aliquot of a dried peanut sample spiked with 150 µg of ethyl parathion and methyl parathion per gram of peanuts was subjected to the outlined procedure. HPLC-ECD conditions as in Figure 5.

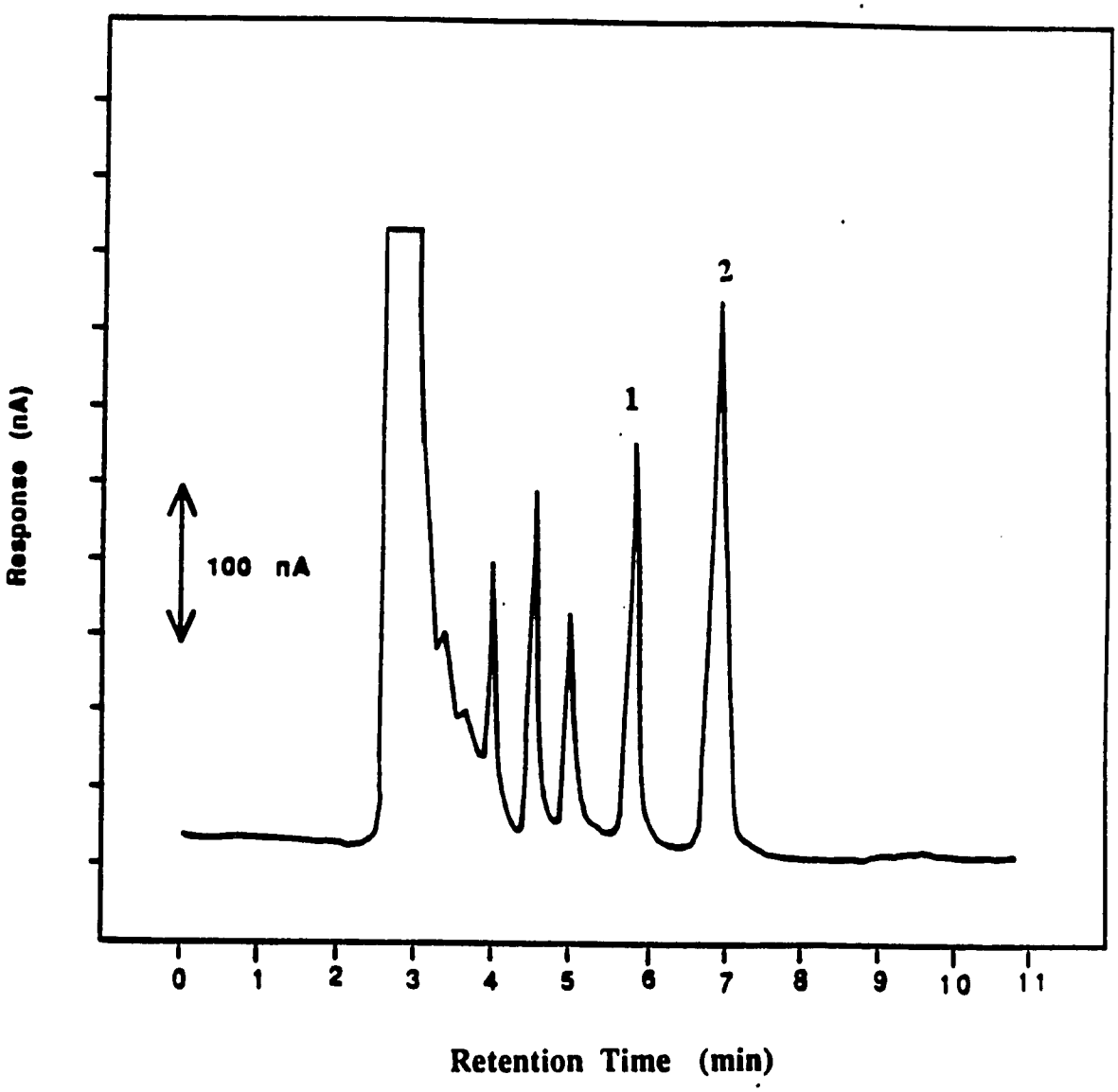

Figure 10. HPLC-ECD chromatogram of a supercritical-fluid extract of a corn sample treated with methyl parathion (1) and ethyl parathion (2). Extraction conditions: SFE with 8000 psi CO_2, 45 ^{0}C, 20 min. A gram aliquot of a dried corn sample spiked with 150 µg each of ethyl parathion and methyl parathion per gram of corn was subjected to the outlined procedure. HPLC-ECD conditions as in Figure 5.

extract used in figure 9 was re-examined with a UV detector at 270 nm and its chromatogram is shown in Figure 11. From this chromatogram, the lack of selectivity and sensitivity of UV detector for extracts containing extraneous materials is evident.

The quantitative results for the four different seeds and grains at three spiking levels are summarized in table 2. Mean percentage recoveries obtained from these crop substrates, fortified at levels from 1.25 μg/g to 12.5 ng/g, were typically greater than 88% and ranged from 68 to 97%. To check the completeness of the supercritical fluid extraction, a fortified sample of rice (12.5 μg/g) was extracted with supercritical CO_2 and then Soxhlet extraction was carried out on the remaining matrix for 24 h with methylene chloride. No additional parathion was found, indicating that SCFE was able to extract the analyte quantitatively at this level of fortification.

Reproducibility

The within day precision was ascertained by analyzing, in triplicate, several samples of rice fortified with methyl and ethyl parathion at three different spiking levels. The results are shown in table 3. The coefficient of variation for both parathions carried through the entire procedure at these fortification levels ranged from 1.9 to 4.8 % (n=3).

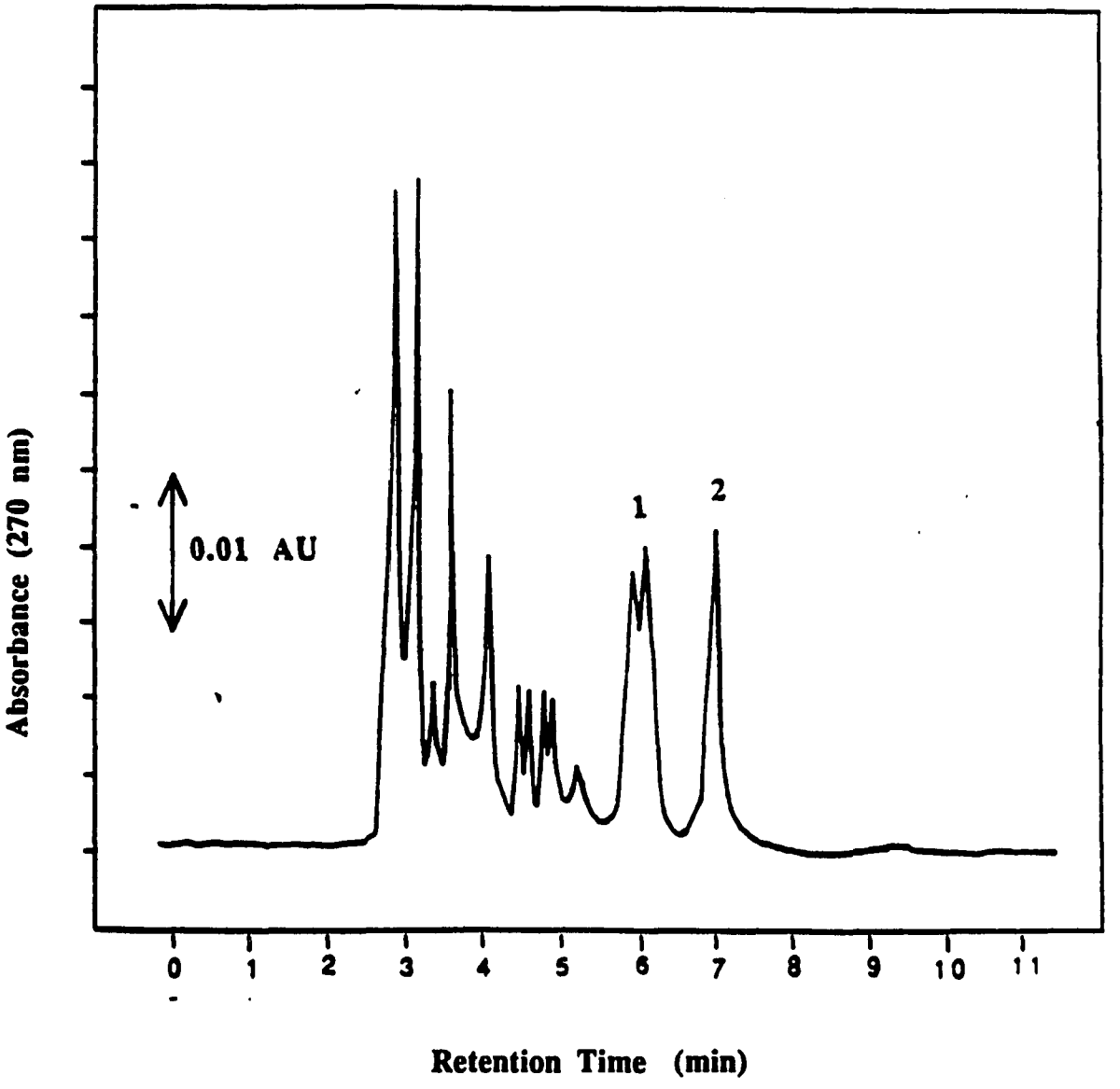

Figure 11. Liquid chromatogram with UV detection of a supercritical-fluid extract of a peanut sample spiked with 150 µg each of methyl parathion and ethyl parathion . Column: 150 mm x 4.6 mm I.D. Supelcosil C-18 (10 µm). Mobile phase: acetonitrile-aq phosphate buffer, pH 6.8 (50+50, v/v). Flow rate was 2.0 mL/min. Detector: Perkin-Elmer 4000 LC system equipped with UV detector at 270 nm. Sensitivity, 0.10 AUFS. Peak identities as follows: 1, methyl parathion; 2, ethyl parathion. Extraction conditions: 8000 psi CO_2 at 45°C, for 20 min.

Table 2. Recovery of parathion from fortified oil seeds and cereals

Substrate	Parathion added (µg/g)	% Recovery	RSD (n=3)
Rice			
	1.25	97	1.9
	0.125	88	3.5
	0.0125	75	7.0
Corn			
	1.25	91	2.4
	0.125	87	3.0
	0.0125	69	5.5
Peanut			
	1.25	95	2.9
	0.125	91	3.1
	0.0125	74	4.9
Sunflower seed			
	1.25	93	2.1
	0.125	89	3.6
	0.0125	68	7.5

Table 3. Reproducibility of SFE and HPLC-ECD method for Methyl and Ethyl Parathion

Amount fortified (µg/g)	Amount extracted (µg/g)	RSD (n=3)
	Methyl Parathion	
25	23.8	3.8
2.5	2.3	1.9
0.3	0.23	4.8
	Ethyl Parathion	
25	25.5	4.1
2.5	2.2	2.1
0.3	0.25	3.9

Chapter 10

Determination of Volatile N-dimethyl and N-diethyl Nitrosoamines in Commercially Available Cured Foods.

INTRODUCTION

A large variety of commercially available cured foods such as sausages, fish, fried bacon and salami contain volatile N-nitrosoamines (1, 2). The presence of nitrosoamines poses a danger to human health because of their potent carcinogenicity and acute toxicity . Thus the isolation and characterization of nitrosoamines has generated a great deal of interest (3, 4, 5) . Their biological and chemical properties are documented in recent reviews (6, 7, 8).
In nature, nitrosoamines are formed under acidic, basic, or neutral conditions by the reaction of nitrite ion with secondary amines with relative ease (9, 10, 11, 12). There is evidence of formation of N-nitrosoamines in foods dried by electric heating, or by hot flue gases or by ovens because of the presence of nitrous oxide in the ambient air (13, 14). The presence of nitrosoamines in the above-mentioned foods at micro gram levels has prompted the development of methods for the isolation and determination of these compounds. Moreover, nitrosoamines have to be isolated from complex matrices. The most commonly used methods include complicated, multi-step sample preparation followed by equally tedious and costly detection methods. (15, 16).
Sen and co-workers directly extracted alkali digested (3M KOH) foodstuffs with methylene chloride, followed by centrifugation and concentration of the organic phase in a Kuderna-Danish concentrator (17). Eisenbrand used a Soxhlet apparatus with methylene chloride for recovering nitrosoamines from dried wheat (18). In the procedure of Kawabata et al., solid foods high in oil content were frozen before extraction with methylene chloride, subsequently the extract was cleaned-up and concentrated (19).

Goodhead and Gough described a versatile steam distillation procedure which is claimed to have universal application to almost any food matrix. The food sample was slurried with a concentrated brine solution , steam distilled, and the distillate extracted using methylene chloride before final clean-up and concentration (20). Libby et al. and Harvey et al. blended processed foods with oil prior to distillation. The distillate, containing all components soluble in the oil-water emulsion and more volatile then oil, was then extracted with methylene chloride and concentrated (21, 22).
More recently, solid phase extraction using Celite as a diluent as well as an adsorbent has become an accepted procedure for isolating nitrosoamines in dried milk products. Harvey et al. applied this method to dried milk. The milk samples were mixed with anhydrous sodium sulfate and Celite. The resulting free flowing dry mixture was packed into a glass chromatography column and the lipid material was eluted with methylene chloride. The extract was further cleaned-up and concentrated (16). Hotchkiss et al. reported a similar procedure for malt beverages (23).

Of all the methods used for the determination of N-nitrosoamines, a TEA detector is most sensitive and selective, but not without very serious drawbacks such as false positive and negative responses, complicated handling and maintenance and a high price (approximately $35,000) (24, 25). Of the other common GC and HPLC detectors only the electrochemical detector has received much attention for these compounds because of its selectivity, sensitivity,simplicity and low cost. Vohra and Harrington described LC separation and polarographic detection of N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA) and N-nitrosodipropylamine (NDPA), with detection limits as low as 800 pg for analytical standards (26, 27) . We have described (Appendix 1) a silver-based thin layer cell capable of detecting 200 pg of readily reducible compounds such as quinones and nitrocompounds. Further improvements in cell design, electrode materials, degassing and pumping hardware, and electronic controlling units will undoubtedly result in selectivity and detection limits comparable to the TEA detector, which approaches 15 ppb for most volatile nitrosoamines, but at a fraction of the cost.

The above methods of extraction succeed to a certain degree towards the goal of isolating nitrosoamines from food samples. However, none of them provides a straightforward, single-step method for quantitative recovery of these compounds with high precision. Supercritical fluid extraction (SCFE) with CO_2, unlike distillation, is performed at low temperature and in the absence of light and other active reagents that might lead to artifact formation or sample loss (22). Supercritical fluid extraction leads to an extract in 15 min. that can be directly subjected to analytical determination without further purification.

In this chapter a supercritical fluid extraction method for isolation of N-dimethyl nitrosoamine and N-dimethyl nitrosoamine from a variety of cured foods, followed by reversed phase HPLC and reductive electrochemical detection at a thin layer silver electrode is described. The performance of SCFE is also compared with a column elution method. Various parameters involved in optimization of extraction, chromatographic separation, and electrochemical detection have been determined.

EXPERIMENTAL

Chemicals

N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA) were purchased from Aldrich (Milwaukee,WI) and used without further purification. Celite 545 was obtained from Fisher Scientific Co. (Pittsburgh, PA). Chloroacetic acid sodium salt was acquired from Kodak. HPLC-grade acetonitrile was obtained from Baker (Phillipsburg, NJ). In-house glass distilled water was used throughout this study.

Precautions

All glassware, spatulas and mechanical tools were scrupulously cleaned to avoid contamination with N-nitrosoamines. Because of the potential carcinogenic hazard, all analytical work was performed, whenever possible, under a well ventilated fume hood using protective gloves. In addition, because of the photosensitive nature of these compounds, solutions were prepared and stored under subdued light.

Preparation of stock solutions of NDEA and NDMA

Fifty five mg of each nitrosoamine was accurately weighed into a 100 ml volumetric flask with a glass stopper, diluted to the mark with acetonitrile and mixed well to form a final concentration of 550 µg/ml. Fresh stock solutions were prepared once a week.

Preparation of working solutions

By serial dilutions of the stock solution, working solutions were prepared in acetonitrile for each nitrosoamine at 55 µg/ml, 5.5 µg/ml, and 550 ng/ml. The dilute solutions were stored in the dark and prepared once a week.

Apparatus

All extractions were performed with a laboratory-constructed high-pressure manifold which has been already described (Chapter 2). In this particular case the collection device was simply an HPLC guard column (4.2 mm x 60 mm) packed with Celite located downstream of the back-pressure regulator. The utilization of Celite facilitates comparison with recommended procedures, which typically employ Celite in the form of a solid phase column.

Chromatography

HPLC with electrochemical detection (ECD) was used to quantify the material recovered from the supercritical extractions. HPLC-ECD analysis was accomplished with a

laboratory-built chromatograph (Appendix 1). In this system, the mobile phase was pumped by a Varian 8500 syringe-pump, modified for continuous mobile phase deoxygenation. Samples, 20 µl in volume, were introduced by a pneumatically-driven delivery device into a Rheodyne 7125 six port valve. Separations were performed on a 10 µm µBondapak C_{18} column 150 mm x 3.9 mm i.d. (Waters Associates, Milford, MA). The electrochemical detector was of the conventional thin-layer amperometric design and employed a silver working electrode, stainless steel auxiliary electrode and a calomel reference electrode (Appendix 1).

Extraction procedure

One gram, accurately weighed, of ground material was transferred to the extraction chamber and held in place by two glass wool plugs. Thereafter, the chamber was inserted into the high-pressure manifold with the downstream pressure regulator opened. After the temperature was brought to 60 ^{0}C, the chamber was purged with low pressure carbon dioxide before the regulator valve was closed. Subsequently, the chamber was pressurized to 8000 psi and equilibrated under these conditions for 15 min. Following equilibration the regulator valve was slowly opened, and the extract was collected on a Celite trap.

Desorption

With a spatula, the extract on Celite was loaded into a 10 ml glass syringe fitted with a coarse frit. The collected N-nitrosoamines were eluted with 4 x 0.5 ml of acetonitrile into a 3 ml Reacti-vial.

Chromatographic conditions

The mobile phase contained 45 vol % acetonitrile in an aqueous 0.01M chloroacetic acid (sodium salt) solution adjusted to pH 3.5 with phosphoric acid; the flowrate was 2 ml/min. The detector was set at -1.2 versus SCE. The sensitivity was set at 100 nA/v.

RESULTS AND DISCUSSION

Hydrodynamic Voltammogram

Various HDVs generated for NDMA and NDEA (Figures 1 and 2) indicated that their reduction waves were irreversible in nature. The general shape of the hydrodynamic voltammograms conformed to those obtained by earlier workers (29-32).

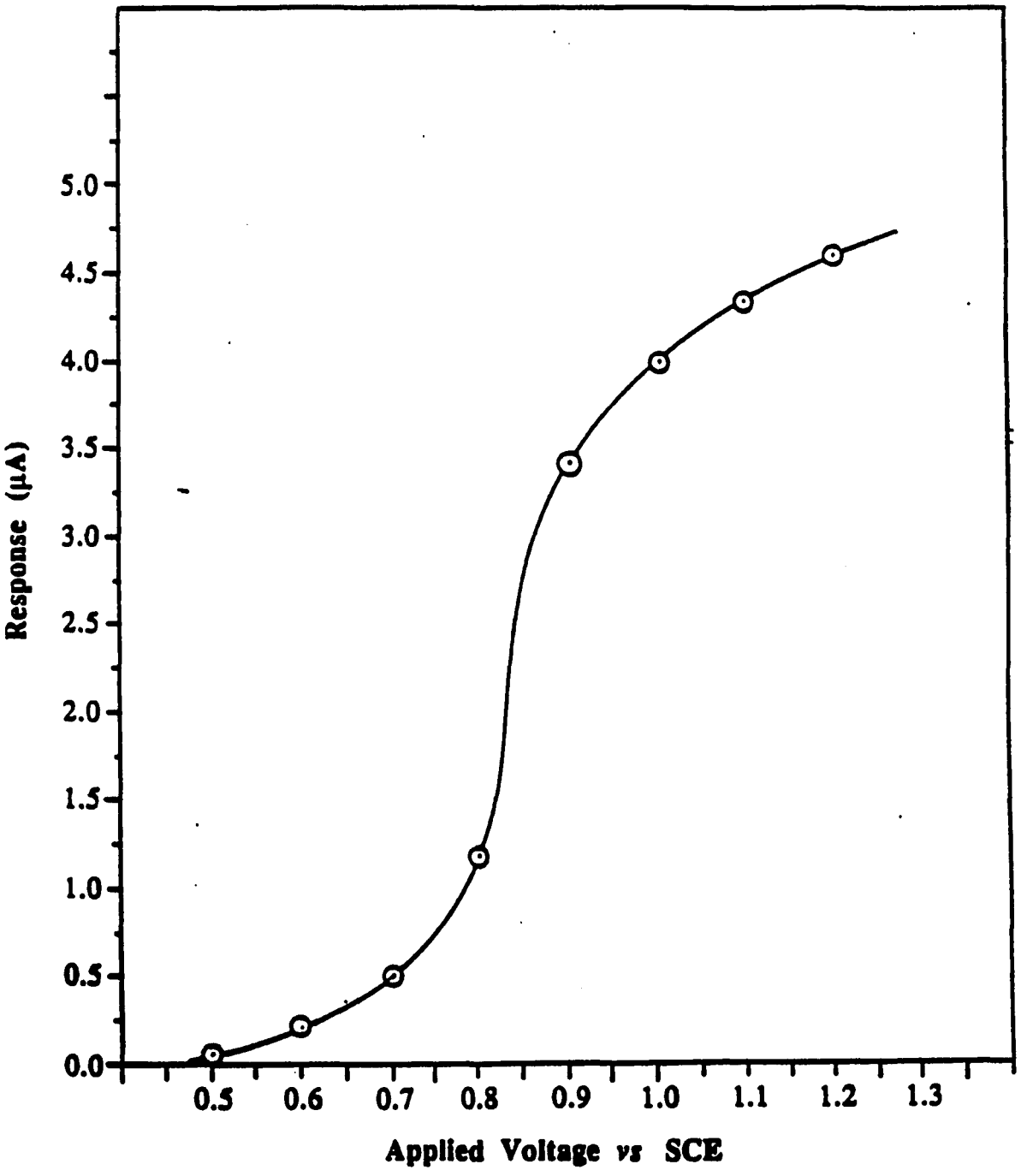

Figure 1. Hydrodynamic voltammogram of N-nitrosodimethylamine (NDMA) on a silver electrode *vs* SCE. Column: 150 x 3.9 I.D. mm μ-Bondapak C-18 (10 μm). Mobile phase: 45% v/v acetonitrile in 0.01M chloroacetic acid (pH 3.5). Flow rate: 2.0 mL/min. Sample size: 0.55 μg NDMA per 20 μL mobile phase injected at each potential setting.

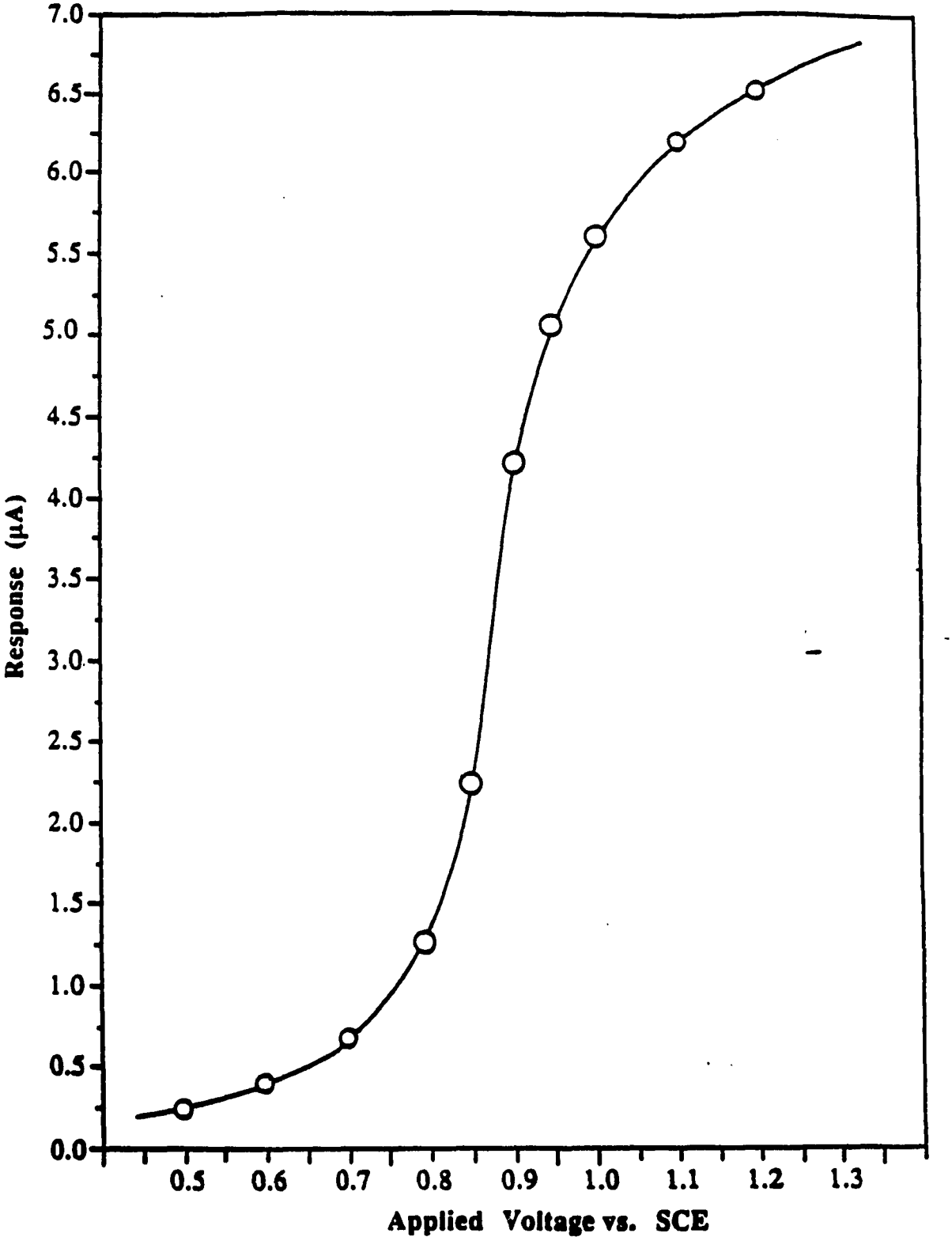

Figure 2. Hydrodynamic voltammogram of N-nitrosodiethylamine (NDEA) on a silver electrode *vs.* SCE. Column: 150 x 3.9 I.D. mm μ-Bondapak C-18 (10 μm). Mobile phase: 45% v/v acetonitrile in 0.01M chloroacetic acid (pH 3.5). Flow rate: 2.0 mL/min. Sample size: 1.1 μg NDEA per 20 μL mobile phase injected at each potential setting.

Effect of pH

The dependence of current on pH is depicted in Figure 3 . Maximum current was obtained at pH of 3.5, hence this value was selected for all subsequent work.

HPLC Analysis

NDMA and NDEA were well separated from each other and from other possibly interfering substances (Figure 4) with retention times 4 and 5 min. respectively, under the chromatographic conditions described above. Figure 5 is a log-log plot of the mean values of the responses obtained from each nitrosoamine at concentrations ranging between 55 µg/mL and 550 ng/mL. An excellent linear response as indicated by slopes of 0.903 and 0.955 nA-mL/ng were obtained for NDMA and NDEA, respectively. Figures 6 and 7 show working curves of peak current versus concentration in the range of 5.0 - 0.5 µg/ml for each nitrosoamine. Both curves were linear over this range with correlation coefficients of 0.996 for NDMA and 0.998, for NDEA. The precision in this range, as measured by the relative standard deviation (RSD), was less than 2.2 %. The minimum detectable quantity for these nitrosoamines is approximately 0.5 ng for a S/N ratio of 3 to 1.

SCFE Extraction

The dependence of percentage recovery on pressure for 1.0 µg/g of NDMA at 60 ^{0}C and 45 ^{0}C from Celite is shown in Figure 8. Figure 9 shows the effect of equilibration time on the efficiency of supercritical fluid extraction. To determine the efficacy of the SCFE procedure for dried milk, 500 ng and 5 µg each of NDMA and NDEA were added to a gram of various dried foodstuffs which had given a zero response for nitrosoamines. The spiked samples were analyzed by the preparation procedure outlined and the recoveries are summarized in Table 1. Typical HPLC-ECD chromatograms obtained for various commodities by this method are shown in figures 10 and 11. The percentage recovery of NDMA and NDEA added to various samples at the two levels are satisfactory and are not significantly dependent on the matrix type.

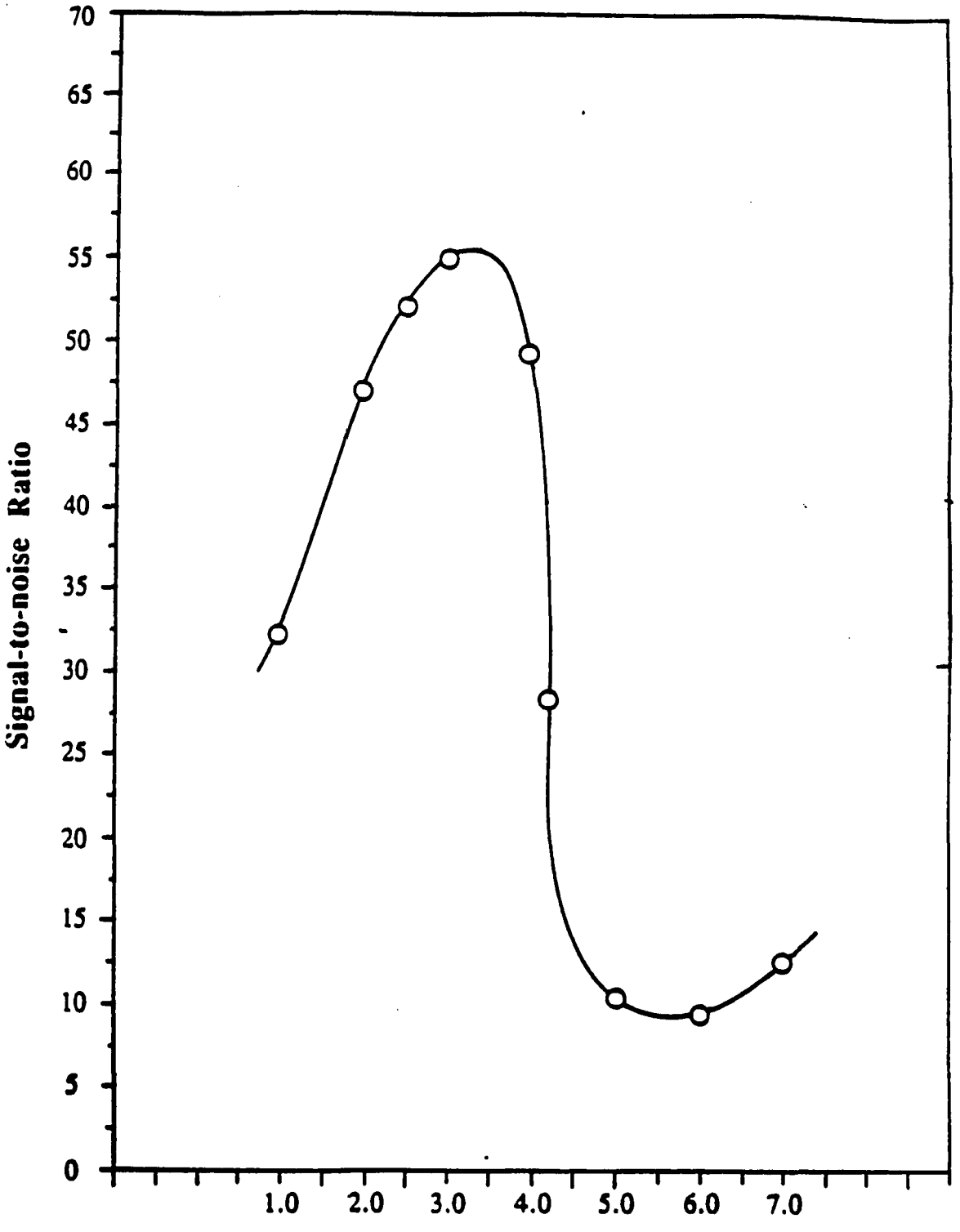

Figure 3. Influence of pH on signal-to-noise ratio for the detection of NDMA at a silver cathode. Chromatographic conditions: column, 150 x 3.9 mm ID μBondapak C-18; mobile phase, 45 % v/v acetonitrile in chloroacetic acid ; flow rate, 2.0 mL/min; applied potential, -1.1 V *vs* SCE.

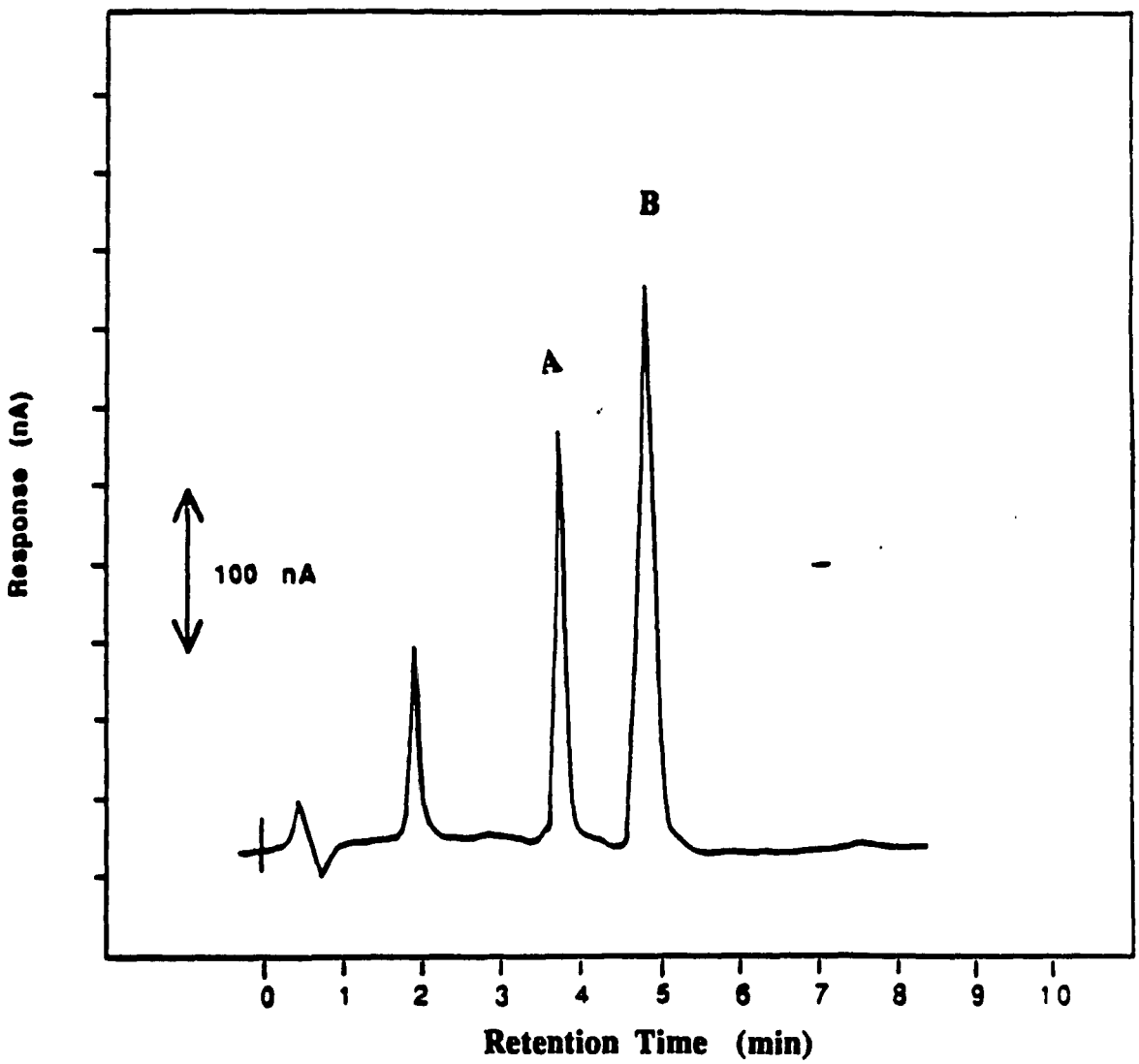

Figure 4. Separation of standard mixture of N-nitrosoamines by reversed-phase chromatography with reductive amperometric detection. Column: 150 x 3.9 I.D. mm packed with μ-Bondapak C-18, particle diameter 10 μm. Mobile phase: 45 % v/v acetonitrile in 0.01M chloroacetic acid (pH 3.5). Flow rate: 2.0 mL/min. Applied potential: -1.2 V *vs* SCE. Peak identities as follows: peak A, NDMA (28.8 ng); B, NDEA (55.0 ng).

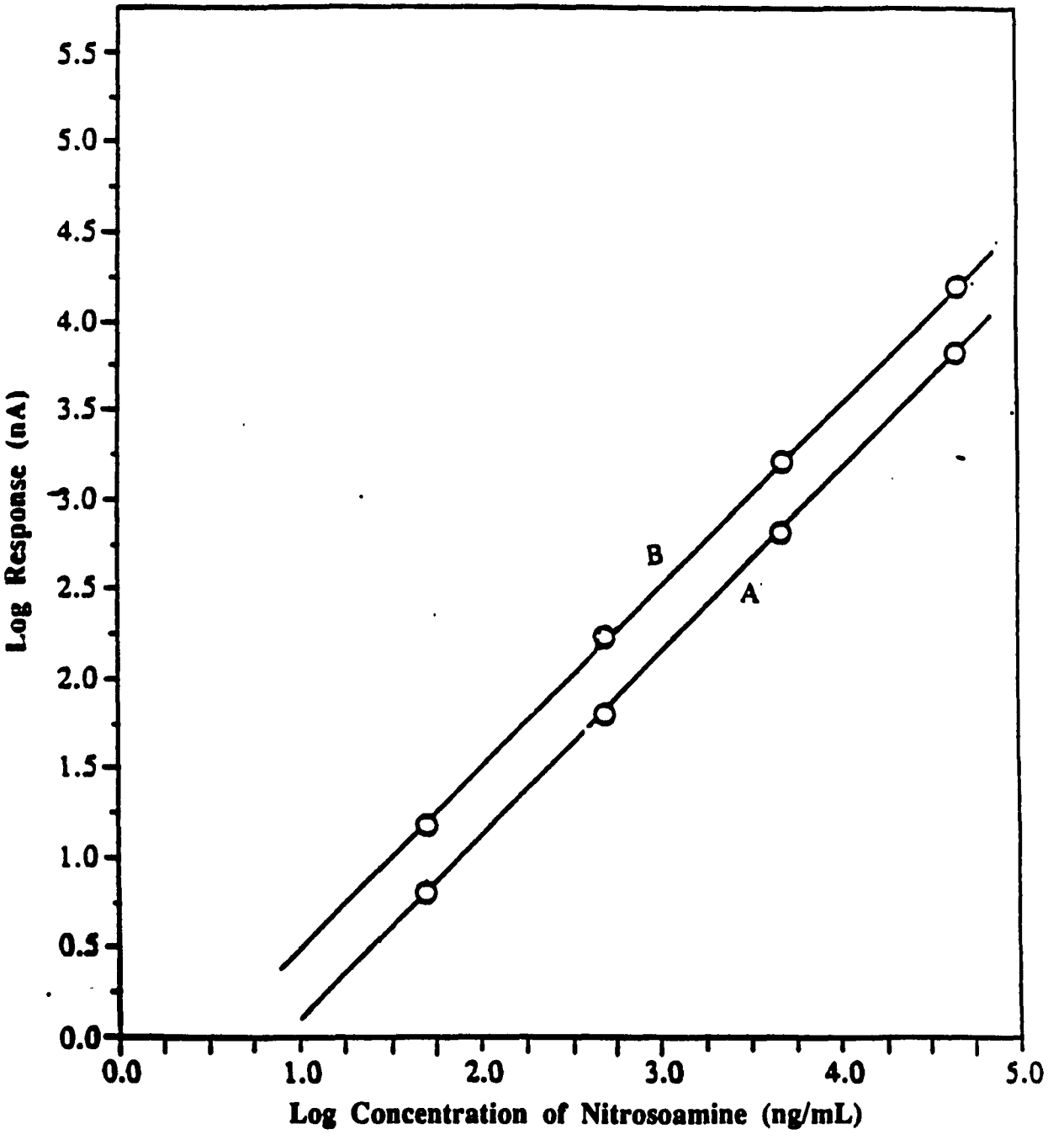

Figure 5. Linearity of detector response to amounts of NDMA (A) and NDEA (B) in 20 µl samples. Chromatographic conditions: column, 150 x 3.9 mm ID µBondapak C-18; eluent, 45% v/v acetonitrile in 0.01M chloroacetic acid (pH 3.5); flow rate, 2.0 mL/min; potential of working electrode, -1.2 V *vs* SCE.

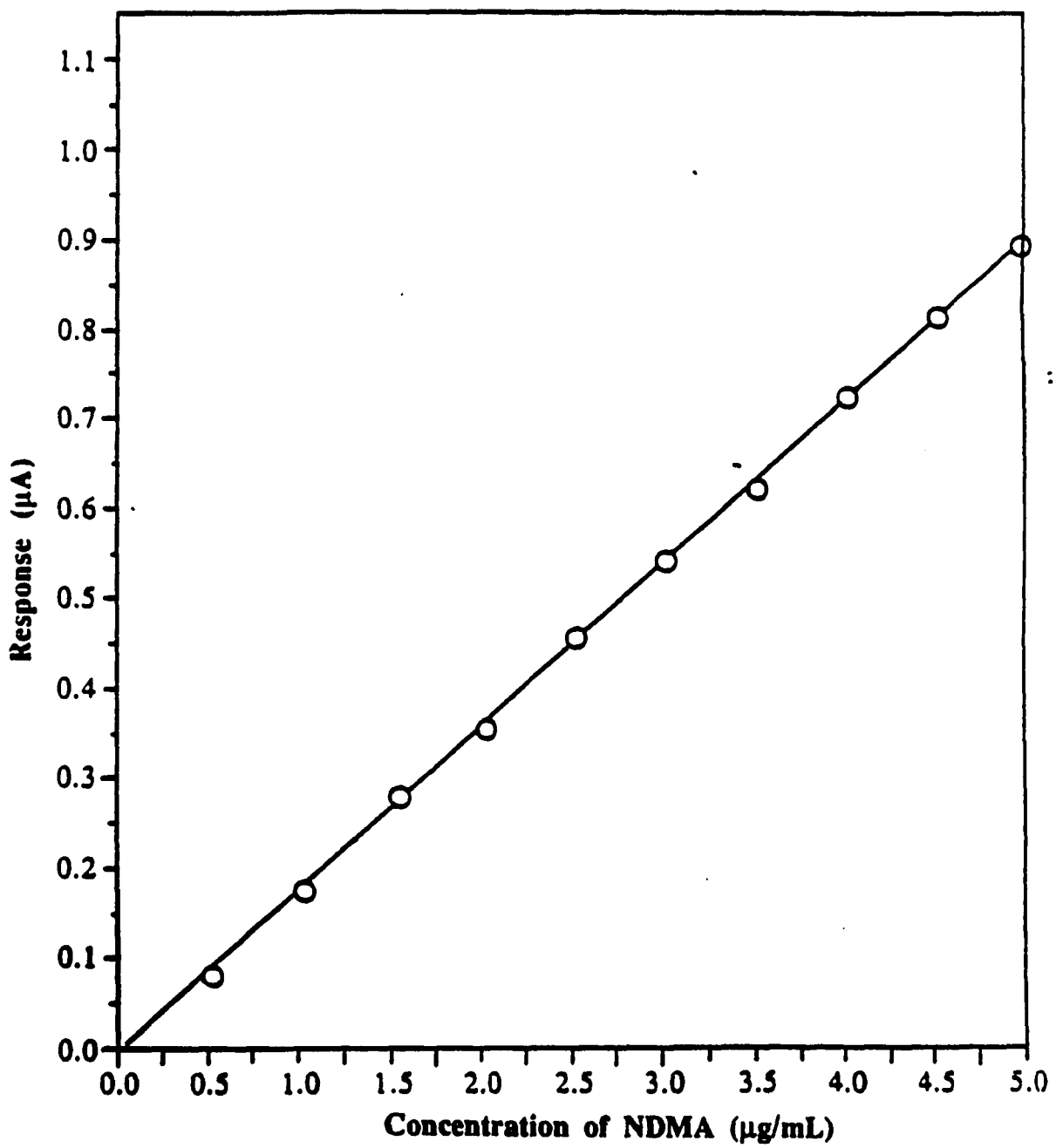

Figure 6. Calibration curve for NDMA obtained from 20 μl injections onto a 150 by 3.9 mm μBondapak C-18 column with a 45% v/v acetonitrile in 0.01 M chloroacetic acid (pH 3.5) mobile phase. Flow rate, 2.0 mL/min. Potential of silver working electrode, -1.1 V vs SCE.

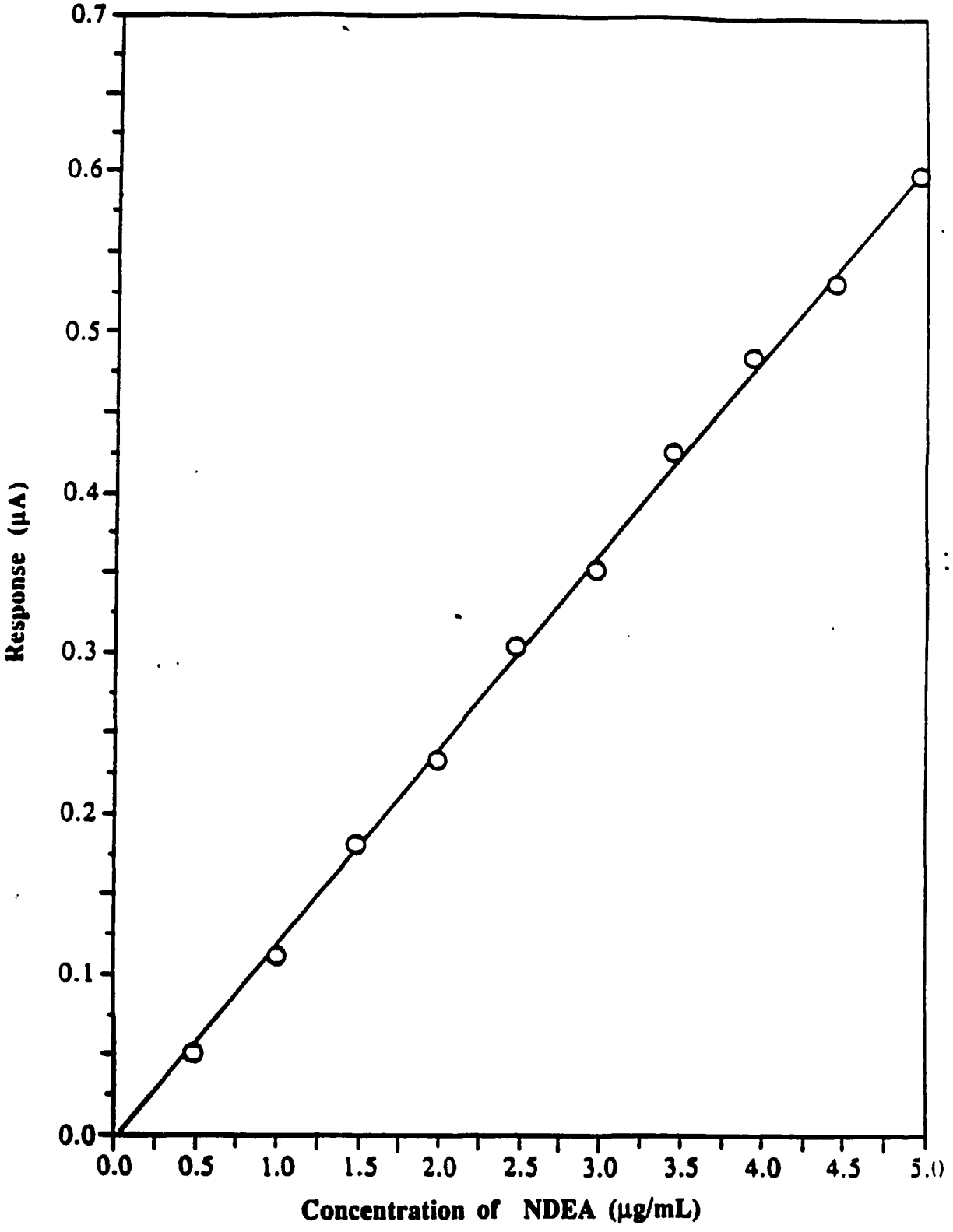

Figure 7. Calibration curve for NDEA obtained from 20 μl injections onto a 150 by 3.9 mm μBondapak C-18 column with a 45% v/v acetonitrile in 0.01 M chloroacetic acid (pH 3.5) mobile phase. Flow rate, 2.0 mL/min. Potential of silver working electrode, -1.1 V vs SCE.

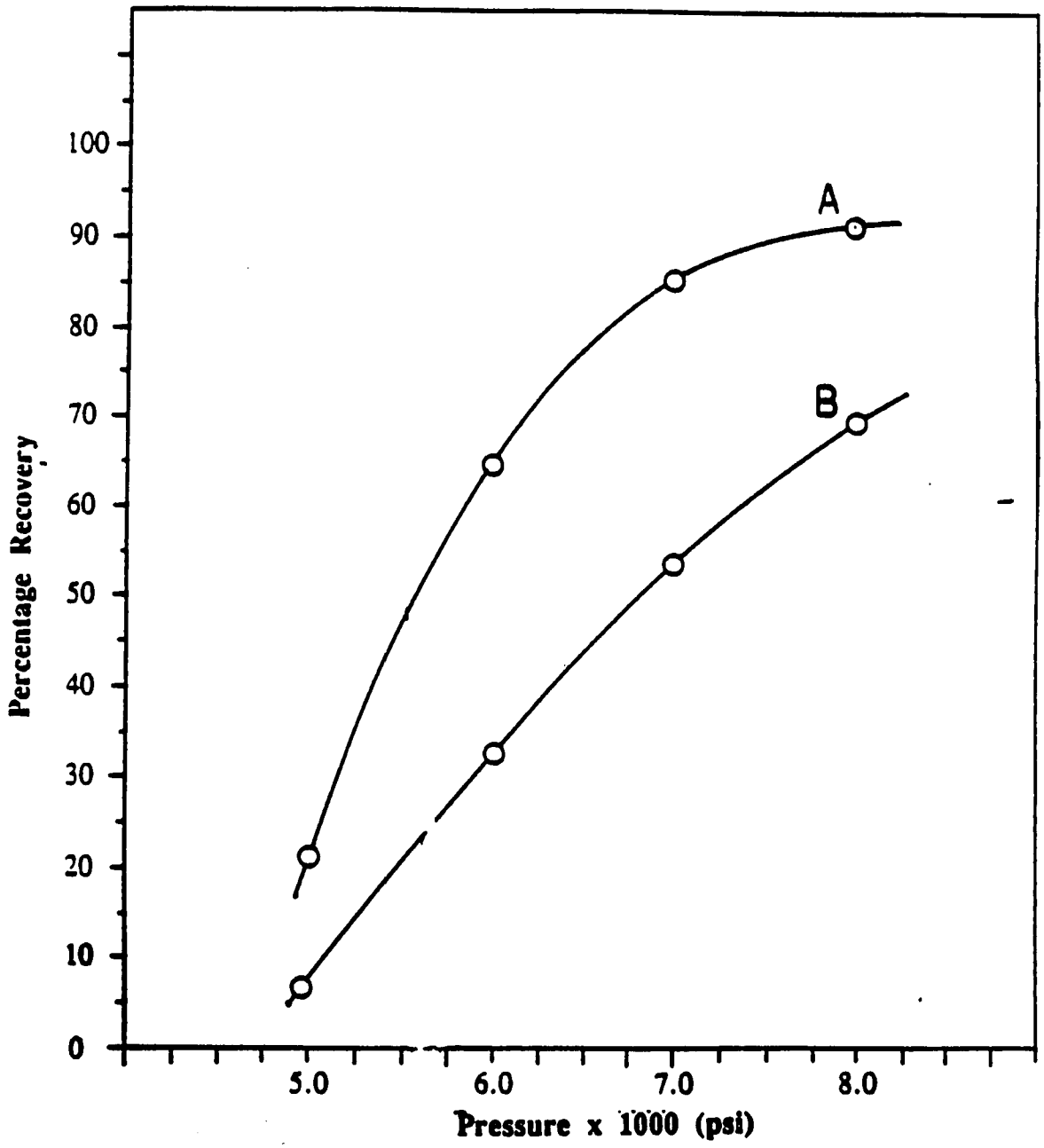

Figure 8. Effect of Pressure on efficiency of SFE procedure for NDMA at (A) 60^{0}C and (B) 45^{0}C. In each case equilibration time was 20 min. One gram samples of Celite fortified with 1 µg NDMA were extracted at each pressure setting. HPLC analysis conditions as in Figure 4.

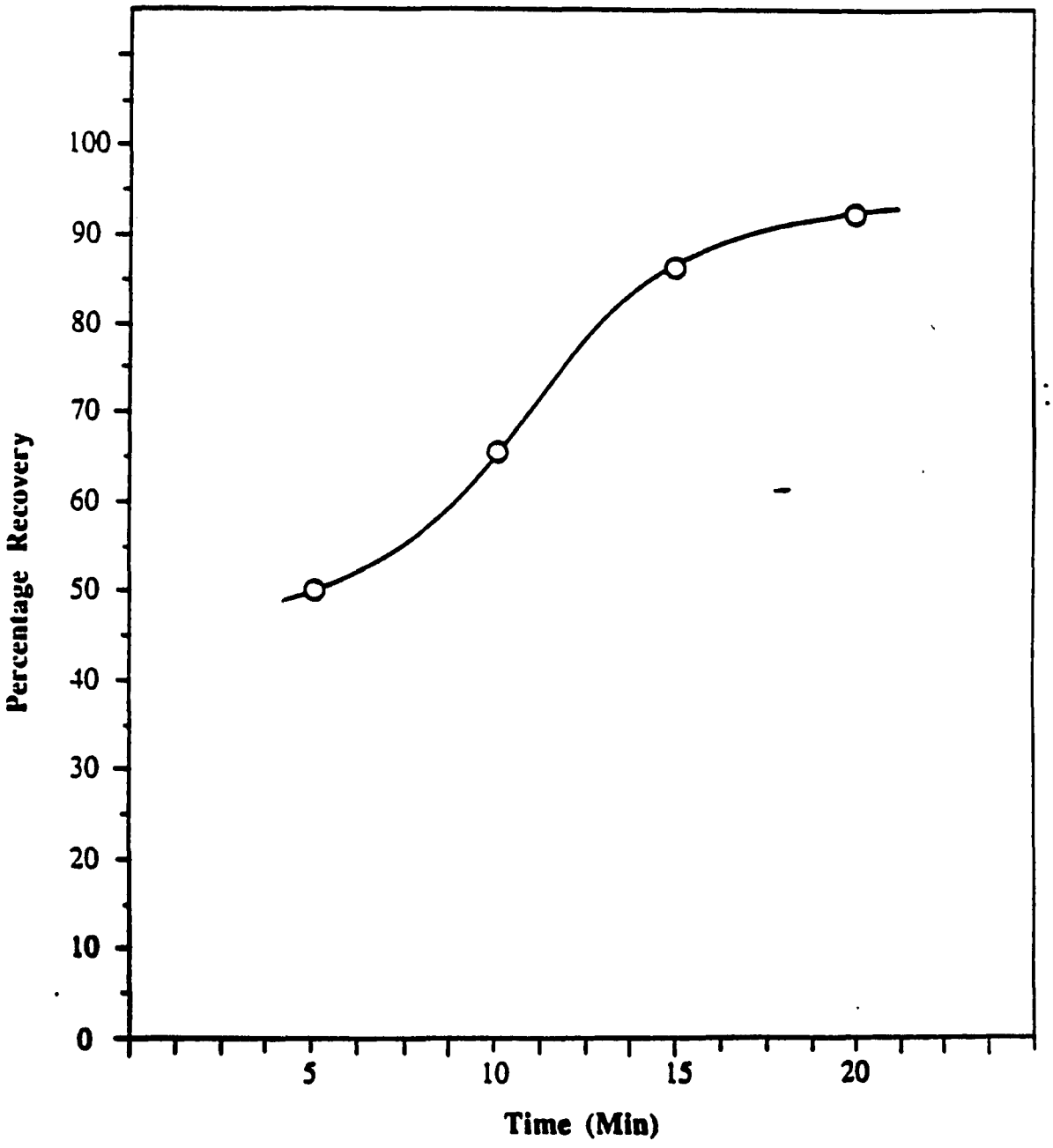

Figure 9. Effect of time on efficiency of SFE procedure for NDMA using CO_2 at 8000 psi and 60^{0}C. One gram samples of Celite fortified with 1 µg NDMA were extracted for each equilibrium time. HPLC chromatographic analysis conditions as in Figure 4.

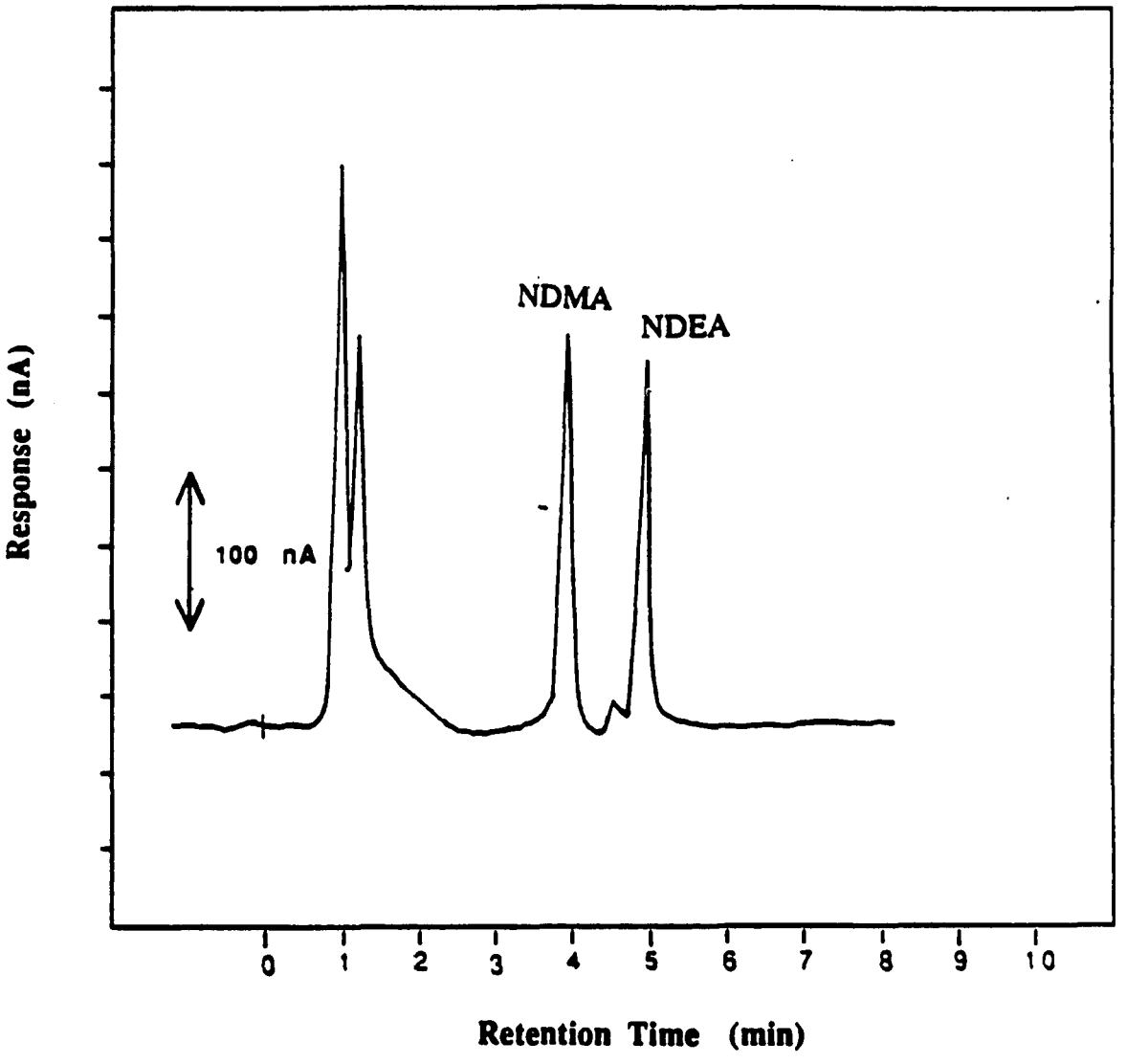

Figure 10. LCEC chromatogram of a supercritical fluid extract of a dried milk sample spiked with NDMA and NDEA. Extraction conditions: SFE with 8000 psi CO_2, 60 ^{0}C, 20 min. A gram aliquot of dried milk spiked with 55 µg each of NDMA and NDEA per gram of milk was subjected to the outlined procedure. HPLC-ECD conditions as in Figure 4.

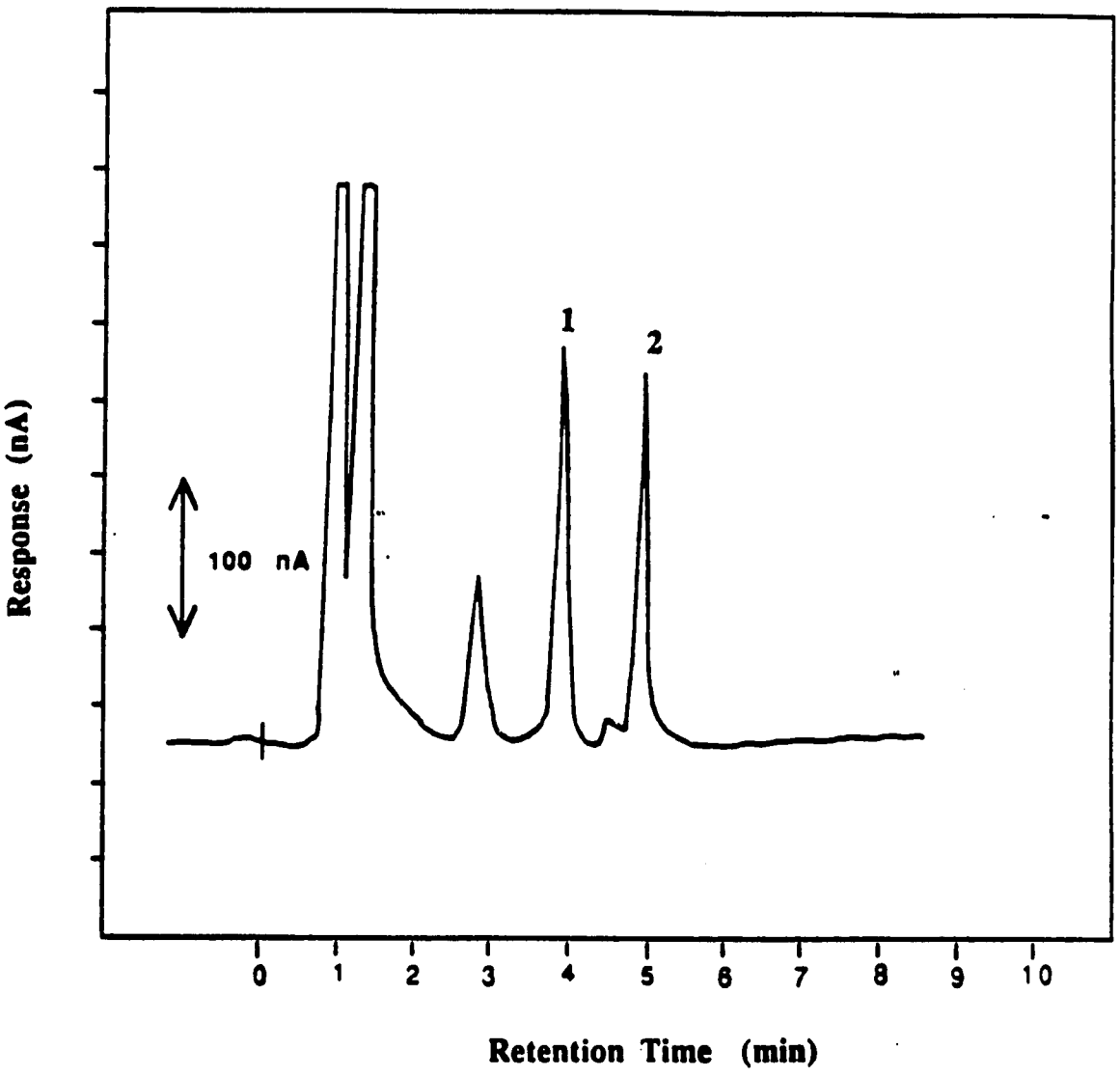

Figure 11. HPLC-ECD chromatogram of a supercritical-fluid extract of an instant coffee sample spiked with NDMA (1) and NDEA (2). Extraction conditions: SFE with 8000 psi CO_2, 60 ^{0}C, 20 min. A gram aliquot of coffee spiked with 55 µg each of NDMA and NDEA per gram of coffee was subjected to the outlined procedure. HPLC-ECD conditions as in Figure 4.

Table 1. Recovery of NDMA and NDEA from fortified foodstuffs.

	Percentage Recovery			
	NDMA fortofication level		NDEA fortification level	
Sample	500 ng/g	5.0 μg/g	500 ng/g	5.0 μg/g
Non-fat milk	89	91	90	93
Chicken soup	83	94	88	95
Coffee	91	93	85	87
Infant formula	86	89	92	94

Linearity of SCFE Procedure

To investigate the linearity of the SCFE procedure, a dried milk sample was spiked with NDMA and NDEA at 5 concentrations ranging from 550 ng/g to 55 μg/g. For each concentration level, 3 replicate injections were made. The dector response was linear at least up to 25 μg/g. A tail-off in response was observed for higher concentrations of nitrosoamines, which was attributed to electrode passivation. Since under normal conditions it is unlikely that concentrations of nitrosoamines over 25 μg/g will be encountered (2, 3), this effect is not regarded as a disadvantage and in any case can be overcome by dilution of the sample. The minimum detection limit for the total procedure approaches 250 ng/g for both NDMA and NDEA (S/N =3).

Precision

The precision of the entire procedure over a normal working day was examined by analyzing 5 replicates of a single sample of milk which was spiked at 550 ng/g. All analyses were performed by the same analyst during the same day. As illustrated in Table 2, the mean concentration recovery was 550 ng/g with a standard deviation of 5.7 ng/g for this sample of milk. Long term repeatability was followed over a period of 3 weeks and involved two determinations per day. The variation of response, 7 %, is higher but the detector will still function adequately at the end of this period. Provided that the detector is calibrated each day with a standard nitrosoamine reference solution, as is normal with any detector, the variations over an extended period cause little trouble.

Interferences

In trace analysis, there is always the possibility of contamination or artifact formation. The potential of the SCFE procedure to form nitrosoamines as artifacts was investigated by adding 50 μl of a 0.10 mg/ml solution of sodium nitrite to a milk sample that had given a negative response for the nitrosoamines and analyzing the mixture. For this particular sample, no NDMA was found in the extract.

Table 2. Within-day Precision of Proposed Procedure of Determining NDMA in Powdered Infant Formula

Determination #	Analytical Result, ng/g
1	544
2	547
3	552
4	557

Range = 544-557 ng/g
Average = 550 ng/g
RSD % = 5.7

Conclusion

Finally, it can be concluded that the methodology of SCFE followed by HPLC-ECD, is comparable to or better than the claaisical methods in terms of recoveries obtained for the volatile, photosensitive N-nitrosoamines. The SCFE procedure is able to provide clean extracts free from problems of sample loss arising from foam formation, thermal or oxidative decomposition, in just 15 minutes. The short extraction times reduce the contact of the worker with toxic and carcinogenic compounds. The highly sensitive and selective electrochemical detector is able to provide a large working concentration range with very low detection limits at a fraction of the cost of a TEA detector.

Chapter 11

ISOLATION AND QUANTITATION OF DIAZEPAM IN COMMERCIAL FORMULATIONS

INTRODUCTION

Diazepam is widely prescribed for symptoms such as anxiety, tension, agitation, irritability, and skeletal muscle spasms. It is manufactured by several pharmaceutical firms and sold under numerous commercial brand names and generic formulations (1).

The advancement in sample preparation methodologies for pharmaceutical formulations in general has not been as fast as in the development of HPLC methods. The efficiency of HPLC methods has been markedly improved by incorporating devices such as automated sample injectors and computerized data stations. The sample preparation methods generally used include, multistep liquid-liquid extraction, and solid phase extraction employing ready-to-use disposable cartridges. The liquid-liquid extraction methods are time-consuming, labor-intensive and suffer from poor recoveries and precision. The solid phase extraction methods though simple, still require significant manual intervention and a time-consuming solvent evaporation step. Recently, pharmaceutical laboratories have employed automated sample preparation methods designed around a robotic arm. The method is cost-effective, but still invovles a multi-step solvent extraction or a solid phase extraction system and hence is prone to errors, poor recoveries and poor analytical precision (1, 2). Moreover, these automated systems are not directly compatible with chromatographic detectors such as mass spectrometers.

Early methods for the determination of Diazepam and other benzodiazepines in pharmaceutical formulations and body fluids relied extensively on GC with an electron capture detector (3, 4). This methodolgy has been reviewed (1, 5-8). Despite the potential sensitivity and specificity of the electron capture detector, elaborate and somewhat lengthy cleanup procedures are necessary and in some cases the high temperature employed in GC may cause the decomposition of metabolites (9, 11). Moreover, derivatization or acid hydrolysis of the more volatile benzophenones may also be required (11). For these reasons and because of the low temperatures used, HPLC would appear to the method of choice for the determination of benzodiazopines and their metabolites. The separation of diazepam and its metabolites by HPLC was first reported by Scott and Bommer (12) using adsorption chromatography for the measurement of diazepam in dog urine. Using adsortption chromatography on silica columns, Weber (13), Bugge (14), Rodgers (15), and Macek and Rehak (16) separated and quantified diazepam and some other 1,4-benzodiazepines in standard mixtures and pharmaceutical preparations. In contrast, Bordie et al. (17) and Ratnoraji et al. (18) used reversed-phase HPLC for the

analysis of Diazepam and its metabolites from various biological matrices. Similarly, the Official Compendia, NFXV and USPXXI (19), also detail reversed-phase HPLC methods utilizing a C-18 column and an aqueous methanolic mobile phase with UV detection at 254 nm for the evaluation of diazepam in tablet, capsule and injectable form. These and other LC methods have been reviewed by Schwartz (20).

Most of the published work on the LC analysis of benzodiazepines has employed UV absorbance detection. However, nearly all of these compounds are electrochemically reducible at practical electrode potentials and their electrochemical behavior has been extensively studied (21-23).

In principle, HPLC with electrochemical detection offers certain advantages over UV detection in terms of selectivity, sensitivity and cost. Also, the construction of a simple flow-through cell and precision potentiostat with low level current measuring capabilities requires minimal time and cost.

The potential offered by SCFE for sample preparation of various pharmaceutical samples, has attracted our interest. A group of veterinary drugs incuding trimethoprim, hexestrol, diethylstilbestrol, and dienestrol have been supercritically extracted and analysed using SCFE-SFC coupled to MS-MS from a freeze dried sample of pig kidney (24).

In this chapter, guidelines are given for the selection of parameters which govern the efficiency of the supercritical fluid extraction and chromatographic components of the over-all procedure.

EXPERIMENTAL

Chemicals

The benzodiazepine reference standard was donated. Commercial tablets were obtained from local distributors. HPLC-grade acetonitrile was purchased from J.T. Baker (Phillipsburg N.J.) In-house glass distilled water was used through the study. Buffer salts were of reagent grade or better.

Preparation of stock solution

Approximately 100 mg of USP diazepam, accurately weighed to 0.1 mg, was transferred into a 100 mL volumetric flask and diluted to volume with acetontrile.

Tablet Preparation

The average weight per tablet was determined and 4 tablets were ground to pass through a 60 mesh sieve. An accurately weighed portion of the composite containing 1 mg of diazepam was transferred to the extraction chamber. The chamber was sealed with two endfittings packed with glass wool and subjected immediately to the proposed SCFE procedure.

Standard Benzodiazepine Mixture

One mL aliquots of the stock solution were transferred to 10 mL volumetric flasks containing 1 mg of Chlorodiazepoxide (Librium)®, and diluted to volume with acetonitrile.

Preparation of Working solutions

By serial dilution of the stock solution, 10 working solutions were prepared in acetonitrile covering the range of 0 to 0.2 mg/mL in 0.02 mg/mL increments.

Apparatus

The HPLC system was based on a Varian Model 8500 syringe-pump, a Rheodyne (Cotati, CA) 7125 rotary injector, and a lab-built electrochemical detector incorporating a silver electrode is described in Appendix 1. The separation was performed on a 10 µm Bondapak C_{18} (Waters Assoc, Milford, MA) column (150 mm x 3.9 mm). The analytical column was protected by a guard column (50mm x 4.6mm) obtained from Supelco (Bellefonte, PA) and dry-packed with pellicular C_{18} bonded silica (40 µm). Compounds were eluted with a mobile phase composed of acetonitrile and aqueous phosphate buffer, pH 6.8. The mobile phase was filtered through a 0.45 µm Millipore filter (Bedford, MA) and degassed with helium for 5 min before use.

Extraction

The chamber with its contents was inserted into the high-pressure manifold (Chapter 2) and heated slowly, while the system was purged of oxygen with low pressure carbon dioxide. When the oven temperature reached 60 ^{0}C, the back-pressure regulator was closed and the system was pressurized to 8000 psi. After 15 min, the back-pressure regulator was slowly opened and the extract was collected on a guard column packed with pellicular C_{18} bonded silica. The trap and the interconnecting tubing were washed with methanol-acetonitrile (50/50, v/v), filtered through a 0.45 µm filter (Gelman) and transfered to a 25 mL volumetric flask. The solution was diluted to the mark with

acetonitrile and subjected to HPLC analysis with EC detection.

Determination

A 20 µL aliquot of the degassed extract was injected onto the HPLC. The benzodiazepine peaks were tentatively identified on the basis of retention time. Quantitation was based on peak-height measured with a laboratory-constructed peak detector (Appendix 1). Response factors for each compound were computed daily from the standard curve.

Results and Discussion

Electrochemical Characteristics of Diazepam

The optimum electrode potential for the electrochemical reduction was determined by generating an HDV using a MeCN-aqueous phosphate buffer (60/40, V/V), pH 6.8 mobile phase (Figure 1). For diazepam, a value of -1.2V *vs* SCE was chosen as a compromise between sensitivity and background signal and loss of selectivity.

Influence of pH on Response

The dependence of electrochemical response on pH was studied by recording the plateau currents at various pH values (Figure 2). The desired pH values were obtained by using acetic acid and ammonia solutions. Although adequate peak shapes for diazepam can be achieved at pH 4, such a buffer affords relatively poor sensitivity, most likely due to increased background currents. It was found that by increasing the pH of buffer to 7, the sensitivity as well as peak shape can be optimized. In addition, the µBondapak packing of the column is unstable at pH values much above 7.5.

Dependence on Flow rate

As expected, the peak-height response increased with flow rate over the range of 10 to 120 ml/hr. The profile is shown in Figure 3. When the data are replotted on a log-log scale, a linear curve is obtained with a slope of 0.39. This value is in good agreement with the theoretical characterization of a thin-layer cell, for which a value of 0.33 is expected (25).

Linearity and Calibration of EC Detector

The linearity of the EC system for Diazepam was found to extend over 3 orders of magnitude from 0.02 µg/mL to 0.2µg/mL, with a correlation coefficient of 0.995. Samples containing Diazepam were determined from a calibration curve by measuring peak heights. The calibration graph shown in Figure 4 was constructed from a set of ten standards covering the range of 0.02 to 0.2 mg/mL. Each standard was injected twice

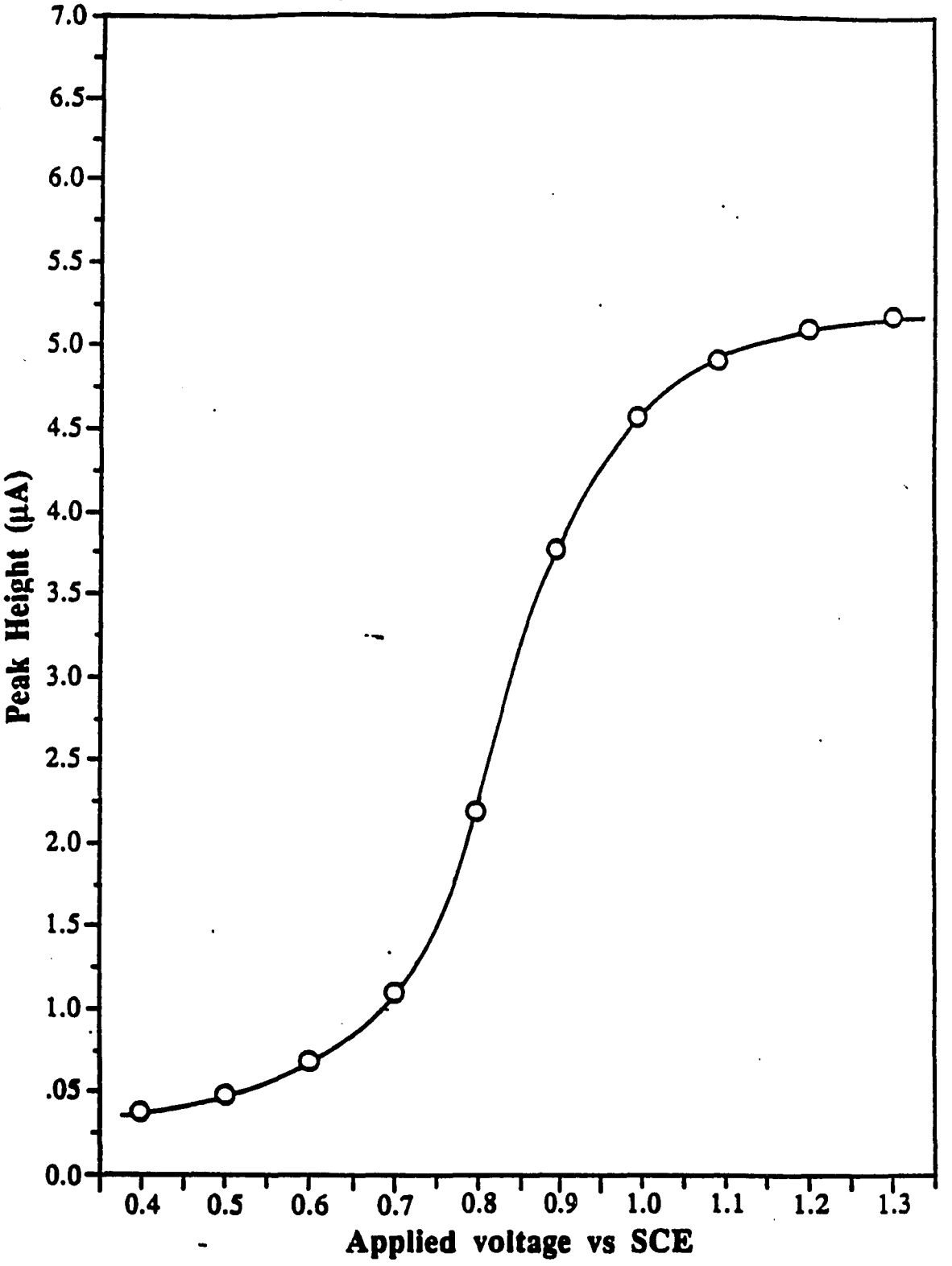

Figure 1. Hydrodynamic voltammogram of Diazepam on silver cathode. Chromatographic conditions: column, 150 x 3.9 mm I.D. µBondapak C-18 (10 µm); mobile phase, acetonitrile-aq 0.05M phosphate buffer, pH 6.8 (60+40, v/v); flow rate, 2.0 mL/min; sample size, 5.0 µg Diazepam in 20 µL mobile phase injected at each potential setting.

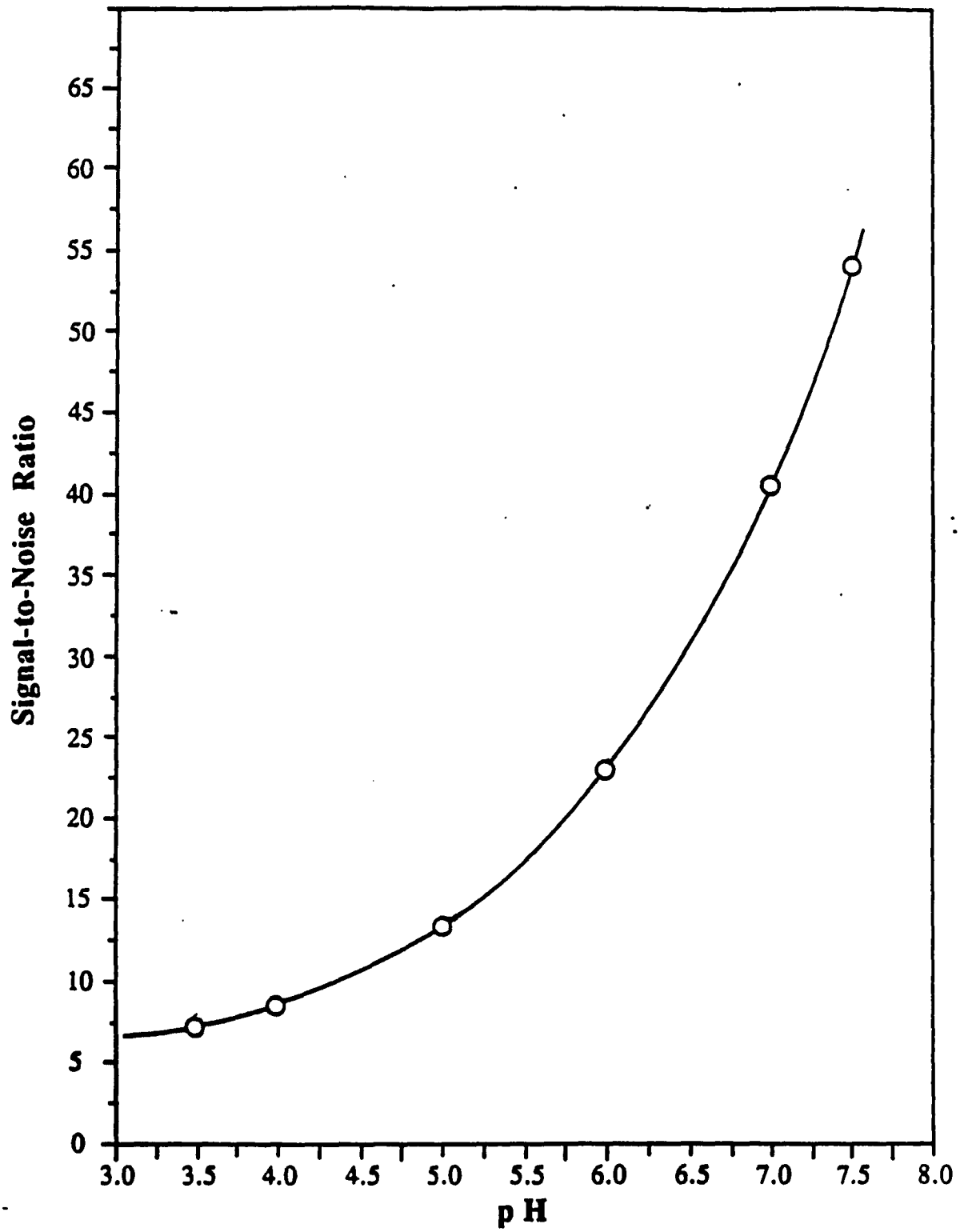

Figure 2. Influence of pH on signal-to-noise ratio in the detection of Diazepam. Chromatographic conditions: Column, 150 x 3.9 mm I.D. μBondapak C-18 (10 μm); Mobile phase, acetonitrile-aq 0.05 M phosphate buffer (pH 6.8); Flow rate, 2.0 mL/min; sample size, 5.0 μg Diazepam in 20 μL mobile phase injected at each pH setting.

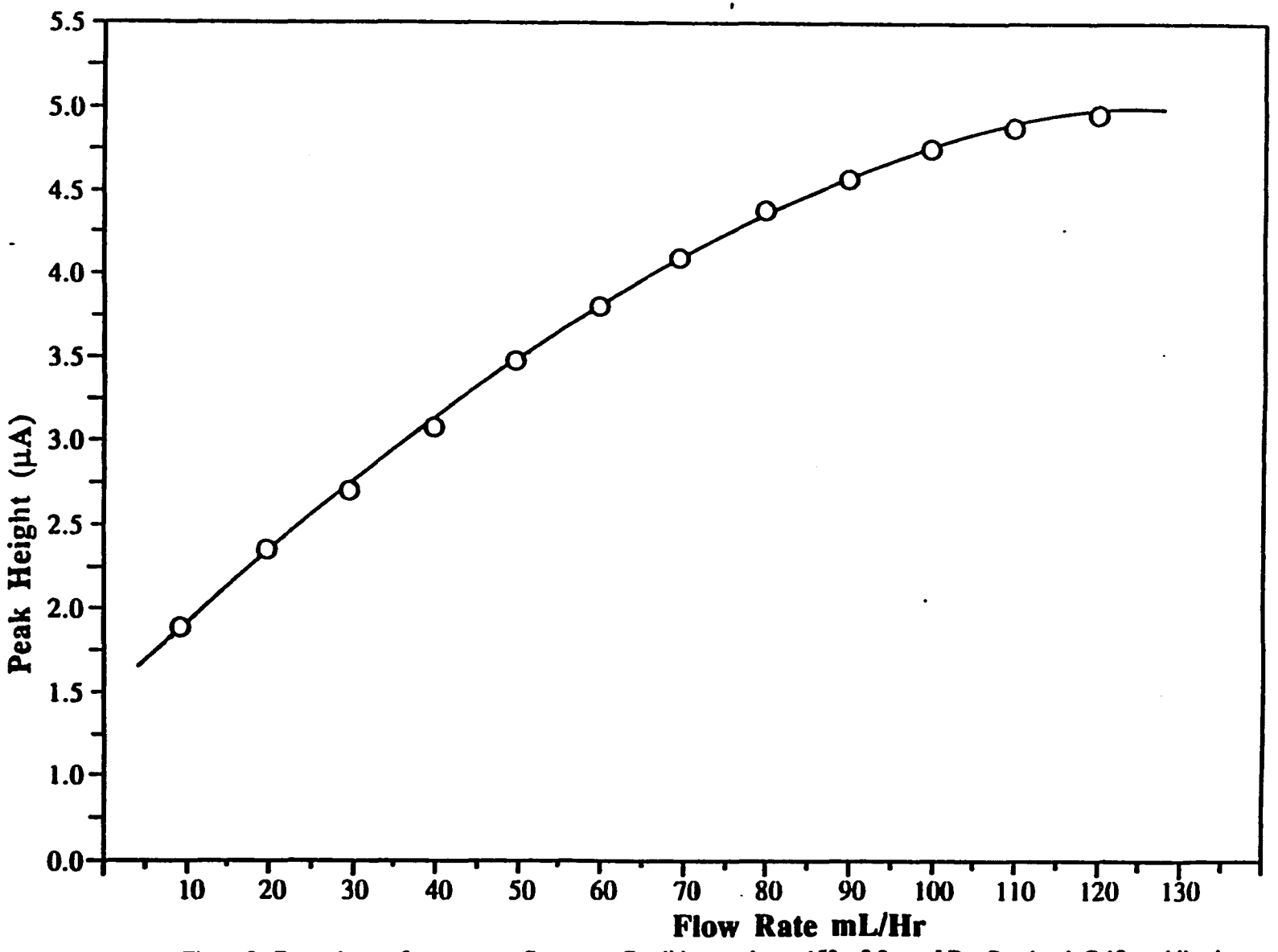

Figure 3. Dependence of response on flow rate. Conditions: column, 150 x 3.9 mm I.D. µBondapak C-18; mobile phase, acetonitrile-aq 0.05 M phosphate buffer, pH 6.8 (60+40, v/v); Ag electrode potential, -1.1 V vs SCE; sample size, 5 µg Diazepam per 20 µL mobile phase injected at each flow rate setting.

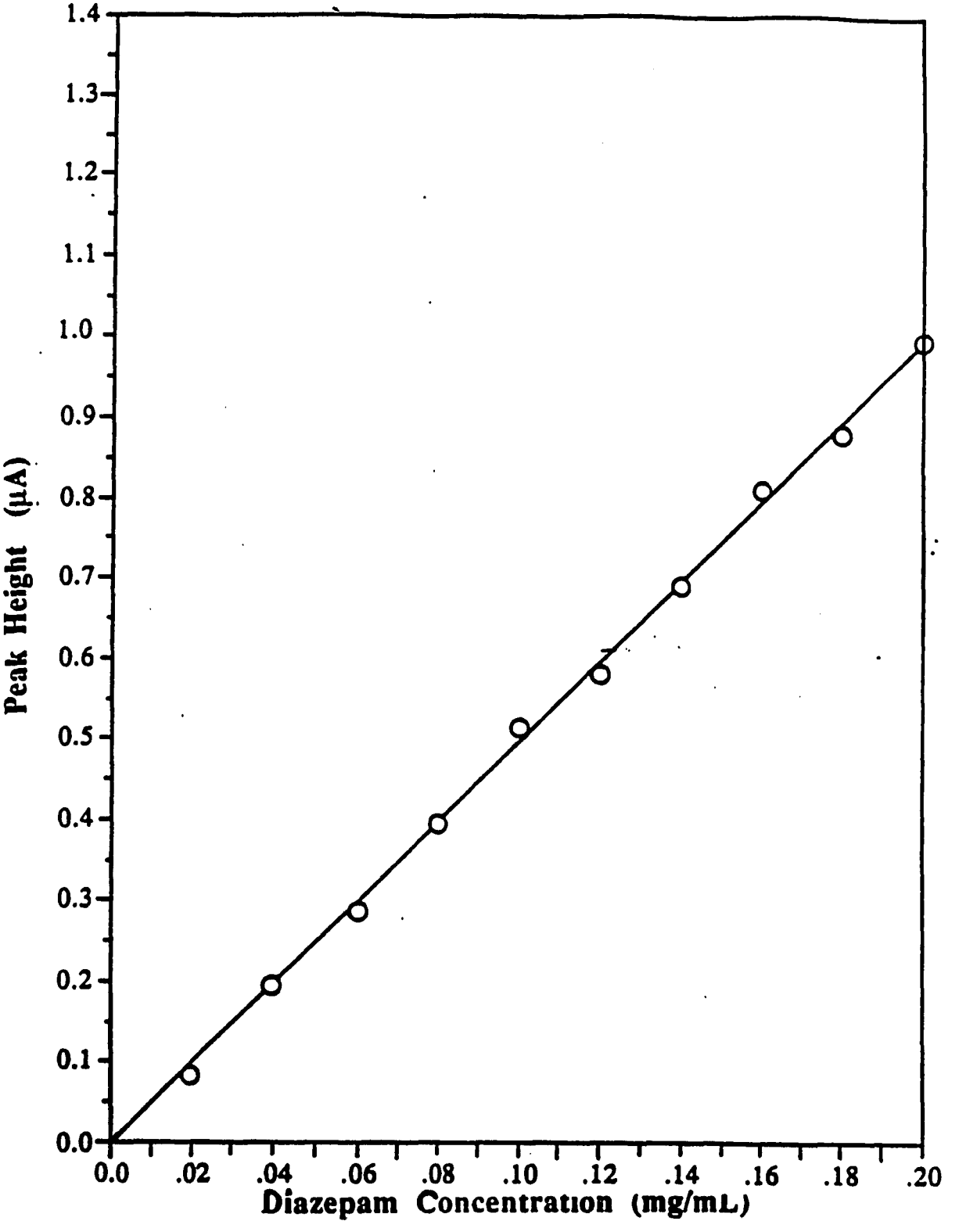

Figure 4. Calibration curve for Diazepam obtained from 20 µL injections onto a 150 x 3.9 mm µBondapak column with an acetonitrile-phosphate buffer, pH 6.8 (60+40, v/v) mobile phase at a flow rate of 2.0 mL/min. Potential of silver working electrode was -1.2 V vs SCE.

and the equation of the best fit line, computed by linear regression analysis, was y (μA) = 5.01 (mg/mL) + .002 with a correlation coefficient of 0.996.

Detection Limit and Sensitivity

Under the optimum chromatographic conditions described above, the detection limit for Diazepam at silver, based on a signal to noise ratio of 3, was found to be 750 pg. The routinely observed blank reading varied between 200 and 250 nA. The sensitivity for Diazepam at silver, defined as the slope of the peak-height versus concentration curve is 5.01 μA-ml/μg.

Chromatography

The chromatographic system was primarily designed to offer short run-times and durability particularly for Diazepam at the expense of resolution of the entire class of benzodiazepines. The relationship between the solvent strength (% MeCN) and retention (capacity factor k') of diazepam is shown in Figure 5. An isocratic eluent containing 60 % (v/v) acetonitrile and 35 % (v/v) aqueous 0.05 M, pH 6.8 phosphate buffer was found suitable for the separation of diazepam from excipients. Figure 6 shows a representative chromatogram of a synthetic mixture composed of Diazepam (Valium) and chlorodiazepoxide (Librium). It can be seen that Librium and Valium show satisfactory peak shapes with retention times of 5.2 and 6.5 min, respectively. An advantage of using this mobile phase in routine quality control situations is that there is a limited dependence of retention time on small changes in mobile phase composition. A change of 5% vol acetonitrile content resulted in only a 0.2 min change in the retention time for diazepam.

Extraction

The extractability of diazepam was characterized by performing repetitive extractions on similar samples using two supercritical fluids at several pressures. Figure 7 illustrates the dependence of percentage recovery on pressure for diazepam using CO_2 and N_2O. From the curve obtained by using CO_2, it can be seen clearly that recovery is not quantitative for an equilibration time of 15 min. On the other hand, quantitative recovery is obtained with N_2O at 8000 psi and 65 ^0C within 15 min. These conditions were used for all further work. The differences in the extraction efficiences of supercritical CO_2 and N_2O are probably due to the polar nature of the analyte and the extractant i.e., N_2O.

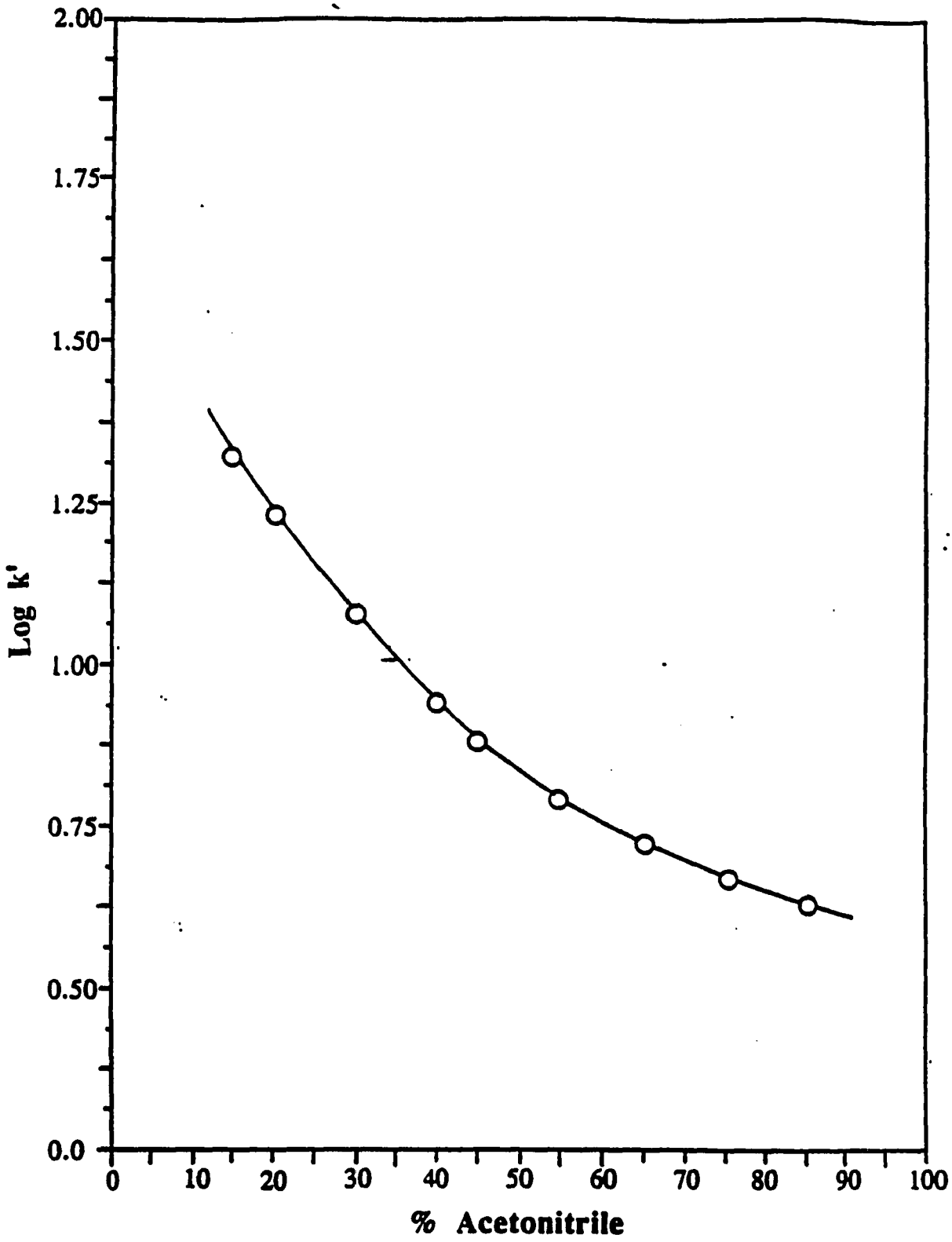

Figure 5. Effect of solvent strength on the retention of Diazepam. Chromatographic conditions: column, 150 x 3.9 mm I.D. μBondapak C-18 (10 μm); mobile phase, prepared from appropiate volumes of acetonitrile and a stock aqueous phosphate (pH 6.8) buffer; flow rate, 2 mL/min; test sample, 5.0 μg Diazepam per 20 μL mobile phase injected at each mobile phase composition.

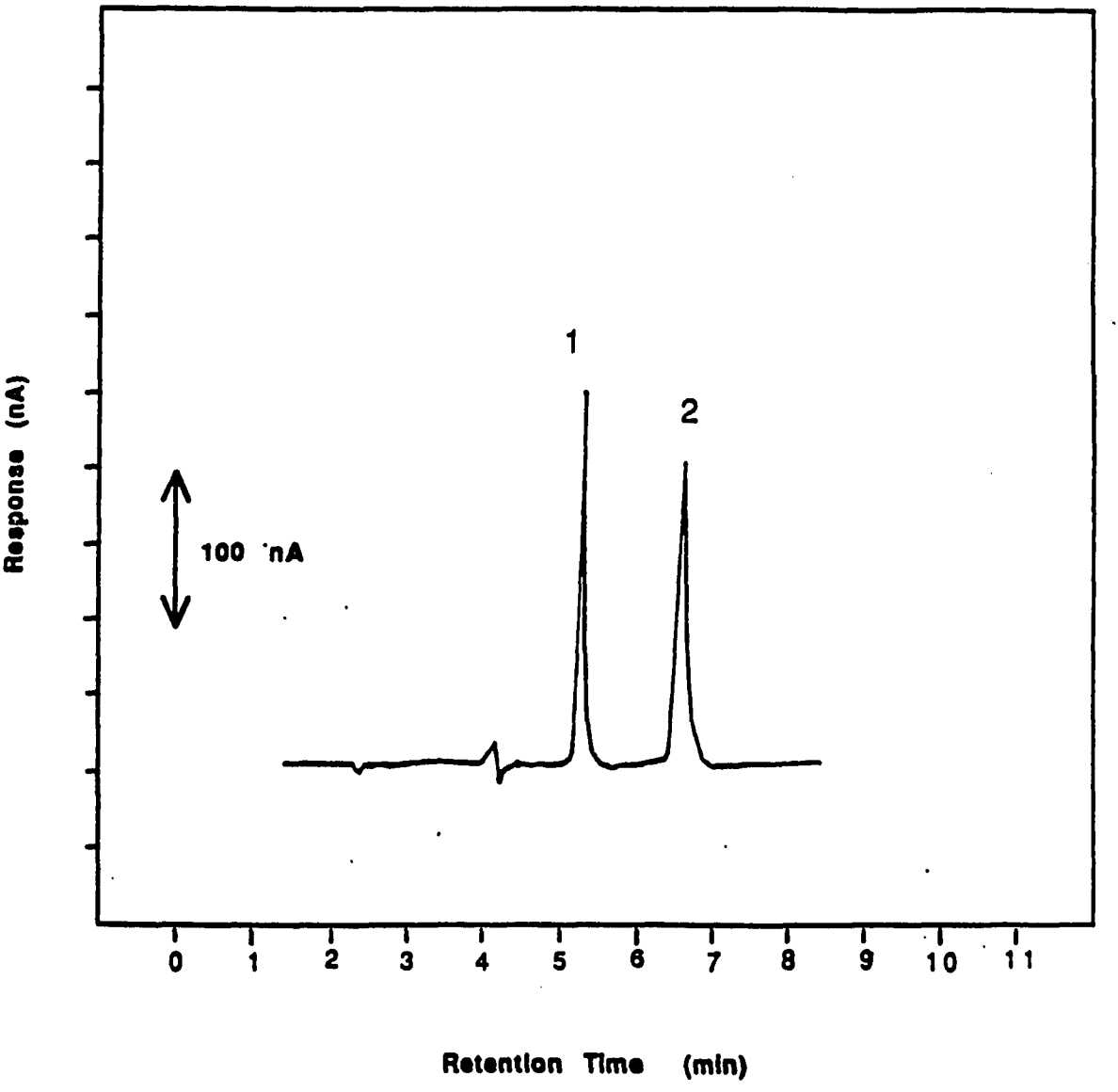

Figure 6. Chromatogram of the separation of benzodiazepines on a μBondapak C-18 (10 μm) column by isocratic elution: mobile phase, acetonitrile-phosphate buffer, pH 6.8 (60+40, v/v); flow rate 2.0 mL/min; detector potential, -1.2 V vs SCE; detector sensitivity 100 nA/V. Peak identities as follows: 1, diazepam; 2, chlorodiazepoxide.

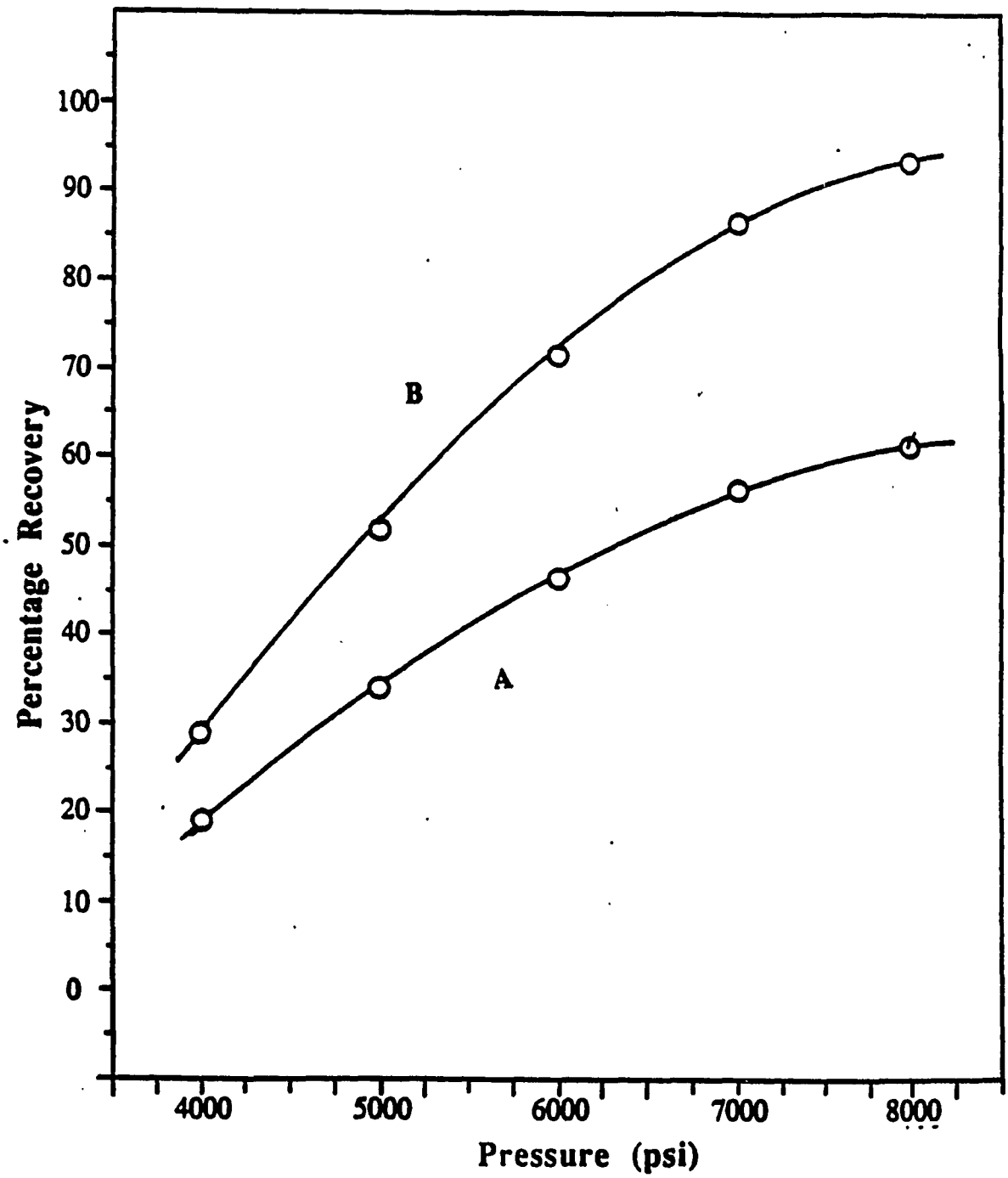

Figure 7. Effect of pressure on efficiency of SFE procedure for Diazepam using (A) CO_2 and (B) N_2O at 65^{0}C for 20 min. At each pressure, one gram samples of Chromosorb W fortified with 100 µg of Diazepam were subjected to the outlined procedure. Chromatographic conditions: column, 150 x 3.9 mm I.D. µBondapak C-18 (10 µm); mobile phase, acetonitrile-aq 0.05M phosphate buffer, pH 6.8 (60+40, v/v); flow rate, 2.0 mL/min; applied potential, -1.1 V *vs* SCE.

Collector

The nature of the trap used to accumulate the extracted diazepam plays an important role in determining the over-all efficiency of extraction. The relative ease of precipitating Diazepam onto a solid support enabled use of a very short (40 mmx 4.2mm) column packed with C_{18} Corasil (Waters Assoc., Milford M.A.). Figure 8 shows a desorption profile for this particular C_{18} trap as a function of MeOH-MeCN (50/50,v/v) volume. It is seen that the first 5 mL removed 75 % of the Diazepam from the trap, and an additional 5 mL of eluent was sufficient to achieve greater than 93 %. The efficiency of the described sample preparation procedure was examined by adding known amounts of Diazepam dissolved in methanol to C_{18} cartridges. After the methanol evaporated, the diazepam was recovered by the SCFE procedure described earlier. The recoveries of the diazepam standards were then measured in triplicate using the electrochemical detector following following reversed-phase separation. The results are summarized in Table 1. The recoveries of Diazepam are linear over the concentration range of 500 ng/mL to 1.0 mg/mL.

Influence of Matrix

The effect of matrix type was briefly investigated by spiking one gram samples of an animal feed (Ralston-Purina Co., rat chaw, type 5010-C) and a C_{18} support with 500 μg of diazepam before the SCFE procedure. In agreement with earlier studies , the recoveries for both types of matrices were very similar (i.e., >90%). Figures 9 and 10 show chromatograms of the recovered diazepam from C_{18} and animal feed, respectively. It can be seen that resolution of diazepam from excipients is satisfactory in both cases. The small peak eluting prior to Diazepam in the chromatogram obtained for the animal feed was resumably due to an unknown component in the sample. No such peak was observed in other chromatograms from standards or samples of Diazepam taken through the entire procedure. Based on these results, the sample preparation procedure described may be judged efficient for the extraction of Diazepam.

Application to Generic Formulations

Diazepam tablets available from 3 generic manufacturers were analyzed for drug content, content uniformity and reproducibility by the proposed method. Assay results for commercial 5.0 mg tablets are given in Table 2. As illustated in Table 2, the method gave results that ranged from 3.9 to 5.3 mg of the 5 mg value declared.

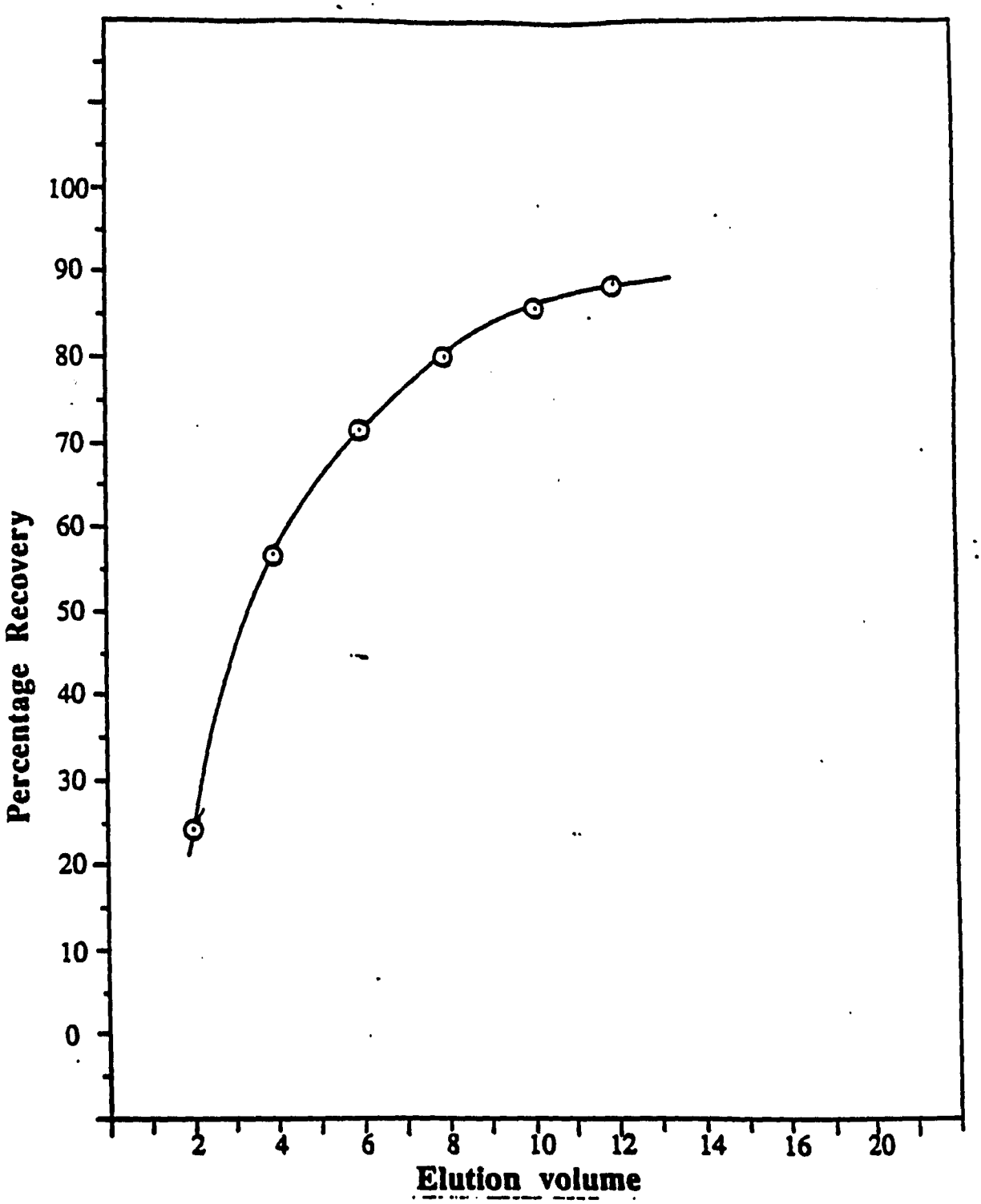

Figure 8. Recovery of Diazepam from C-18 cartridge as a function of eluent volume. Desorption conditions: eluent, methanol - acetonitrile (50+50, v/v). Sample size: cartridge spiked with 100 µg diazepam per gram of C-18 stationary phase. Chromatographic analysis conditions: column, 150 x 3.9 mm I.D. µBondapak C-18 (10 µm); mobile phase, acetonitrile-aq 0.05M phosphate buffer (pH 6.8); flow rate, 2.0 mL/min; detector potential, -1.2 V vs SCE; current sensitivity, 100 nA/V.

Table 1. Recovery of Diazepam From Fortified C_{18} Cartridges Using SCFE approach described

Fortification level (µg/mL)	Recovery, %
1000	91.3
500	92.6
100	94.1
50	93.0
10	88.2
5	93.4
1	90.7
0.5	81.9

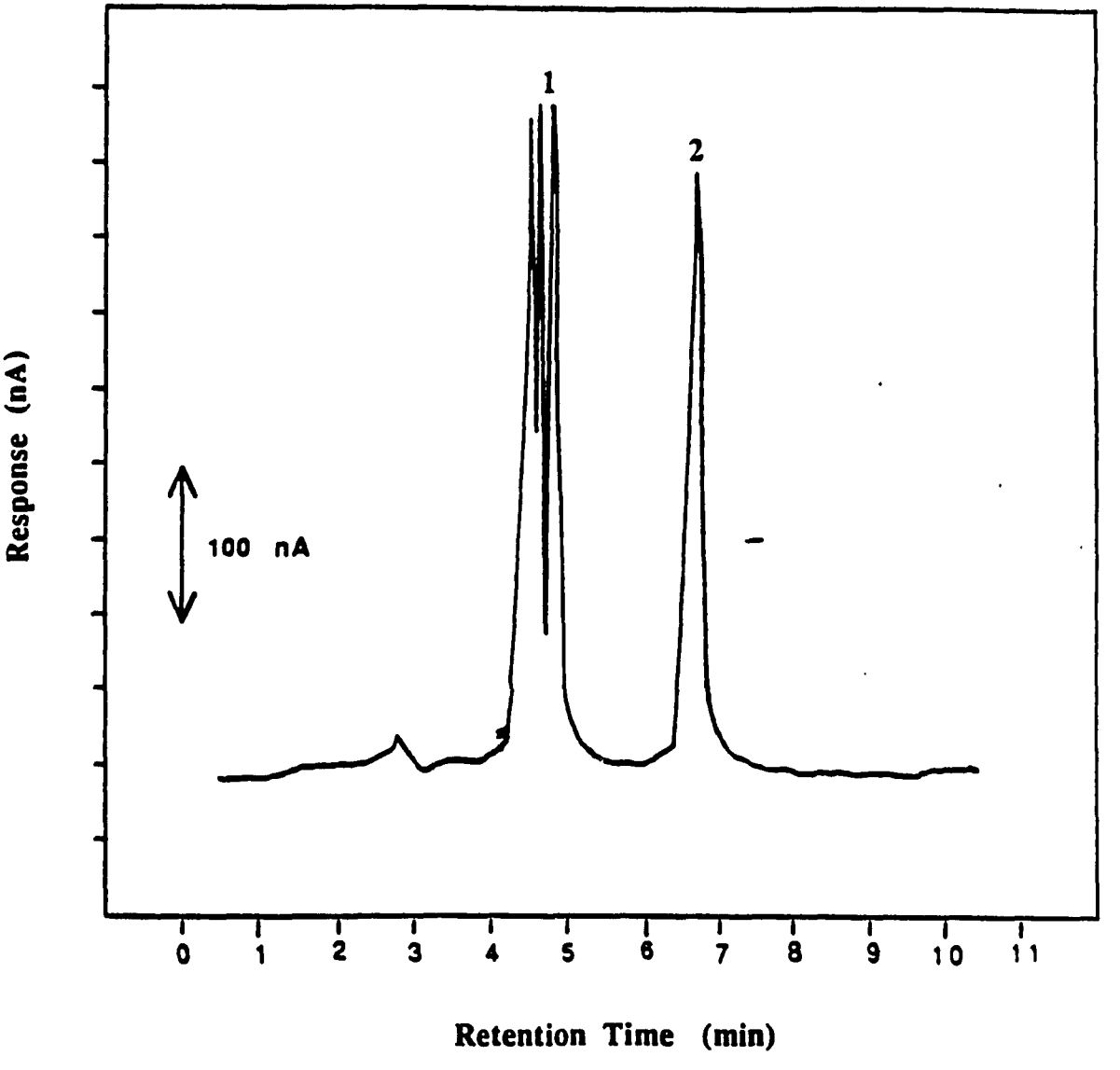

Figure 9. LCEC chromatogram of a supercritical fluid extract of Diazepam from C-18 cartridge. Chromatographic conditions: column, 150 x 3.9 mm I.D. μBondapak C-18 (10 μm); mobile phase, acetonitrile-aq 0.05 M phosphate buffer (pH 6.8); flow rate, 2.0 mL/min; detector potential, -1.2 V vs SCE; current sensitivity, 100 nA/V. Adsorbent cartridge spiked with 100 μg per gram of C-18 packing. Peak identities as follows: 1, impurity; 2, Diazepam.

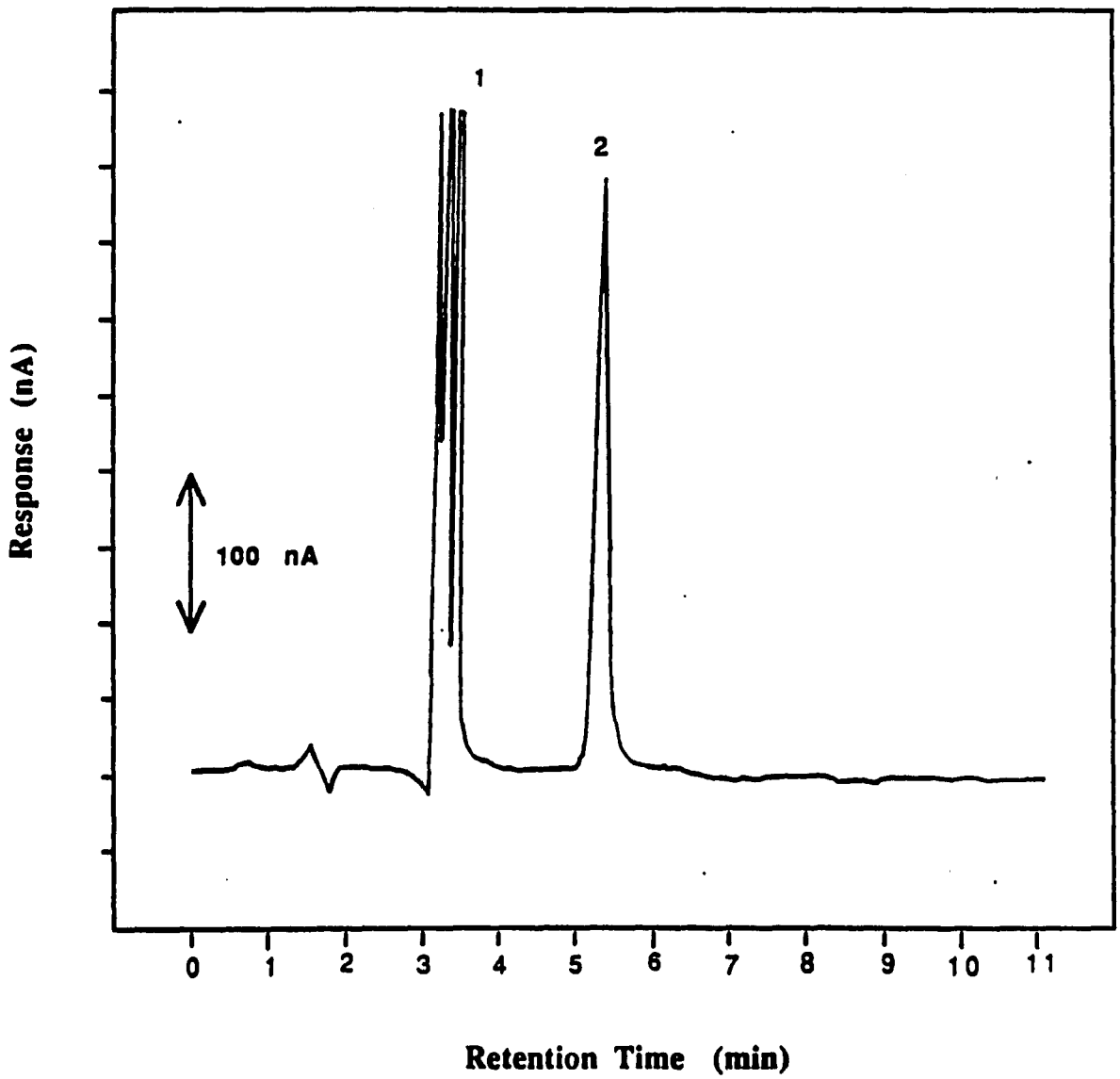

Figure 10. LCEC chromatogram of a supercritical fluid extract of a Diazepam spiked feed sample. Chromatographic conditions: column, 150 x 3.9 mm I.D. μBondapak C-18 (10 μm); mobile phase, acetonitrile-aq 0.05M phosphate buffer (pH 6.8); flow rate, 2.0 mL/min; detector potential, -1.2 V vs SCE; current sensitivity, 100 nA/V. Feed sample spiked with 100 μg per gram of chow. Peak identies as follows 1, impurity; 2, Diazepam.

Table 2. Results of HPLC-SCFE analysis of Commercial Diazepam Tablets

Manufacturer	Found, Percentage of Declared		
	Run 1	Run 2	Average
Hoffmann LaRoche	95	94	95
Durimed	105	103	104
Barr	93	97	95

Content Uniformity

Results of individual tablets taken through the procedure are shown in Table 3. For Durimed brand individual tablets were found to contain on the average of 5.02 mg of the 5.0 mg label claim.

Reproducibilty

The precision of the method was evaluated by performing 4 replicate analyses on a composite sample equivalent to 1/5 of one tablet. As shown in Table 4, the within-day coefficient of variation for diazepam was 1.8 %. The between-day precision in the same range was 4.6 %.

Standard Addition

The performance of the proposed method for the quantitation of diazepam formulations was further tested by standard additions of 1 to 4 mg of diazepam in four equal intervals to a composite diazepam sample. A typical standard addition curve for a composite sample containing 1/5 of diazepam tablet is shown in Figure 11. For this sample, the average diazepam content was 5.07 mg and the recovery over this range was 98.4 %. On a single day, the relative standard deviation (RSD) for the spiked samples remained well below 2.5 % in a well equilibrated system.

Table 3. Content Uniformity Assay Results of the Durimed Brand Diazepam (5.0 mg) tablets

Tablet	Found, mg/Tablet	Found, % of declared
1	5.05	101
2	5.22	104
3	4.94	98.9
4	4.87	97.4
Average	5.02	100
RSD	3%	

Table 4. Replicate Analysis of Diazepam* tablets by SCFE-HPLC.

Sample No.	Diazepam Found	Percentage Claim
1	5.08	102
2	5.14	103
3	5.05	101
4	5.15	103
Average	5.11	103
RSD	0.98	

*** Durimed**

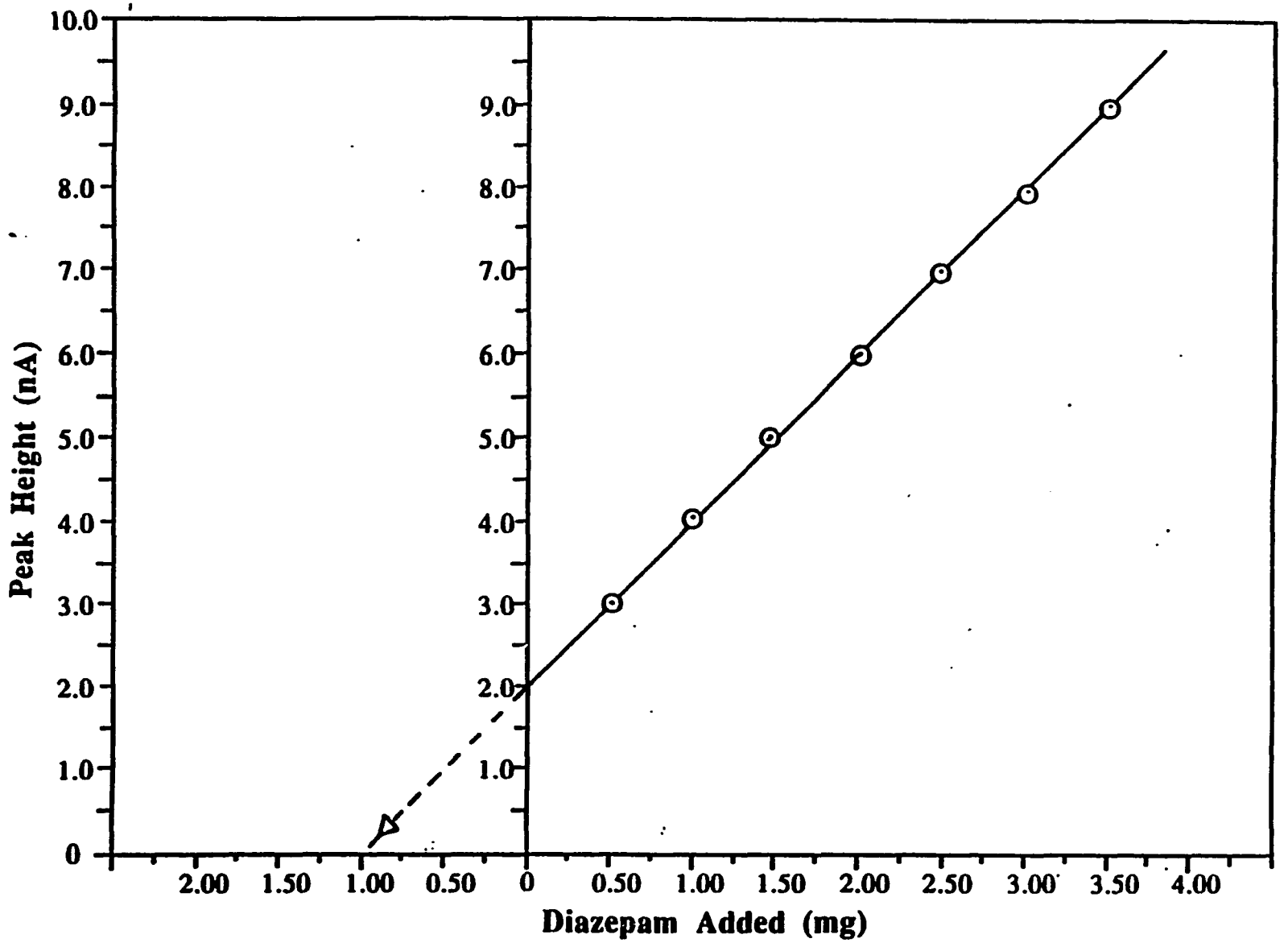

Figure 11. Standard addition curve used in the quantitation of diazepam tablets. Chromatographic analysis conditions as in Figure 6. Each composite sample, equivalent to 1/5 label declaration, is spiked with standard diazepam at increasing increments of 0.5 mg prior to SFE sample preparation.

Conclusion

A fast, accurate, reliable and relatively straight forward method for isolation and quantitation of Diazepam in commercial formulations tablets has been developed. The method incorporates many desirable aspects of previously reported HPLC assays, but reduces the complexity of the sample preparation step by eliminating many manipulation steps, including multiple extractions, transfers, and solvent evaporation. By using SCFE as a quantitative sampling tool, Diazepam can be rapidly extracted for subsequent quantitation with an electrochemical detector.

APPENDIX 1

ELECTROCHEMICAL DETECTOR

ELECTROCHEMICAL DETECTOR

A schematic diagram of the detector cell desinged by Schneiderman et al. (1) is shown in Figure 1. The cell is composed of two sections, one containing the working and auxiliary electrodes, and the other reference electrode. The former is constructed of two 1 in. x 1 in. x 1/2 in. thick blocks separated by a 0.005 in. thick PTFE spacer gasket into which is cut a slot 0.75 in x 0.156 in. wide. The slot defines the 9.6-µl cell volume. The silver electrode is located towards one end of the slot. The top block is made of Type 304 stainless steel and serves as the auxiliary electrode. Column effluent flows into the cell through a 0.025 in. hole in the top block and out to the reference electrode compartment through a 0.043 in. hole in the same block. The column-to-detector inlet connection is made with a 1/16 in. PTFE tubing using Kel-F Parker fittings (10-32, 30^0 tapered).

The silver electrode is a 3/8 in. (length) x 0.125 in. (o.d.), silver rod (4N pure, Hardy & Harmon) force-fit into a 1/8 in. hole drilled into the Kel-F bottom block. Electrical connection to the silver is made with a 0.040 in. Ag- or Au-plated pinjack (Concord Electronics) forced into a hole drilled lengthwise into the center of the silver rod. The block containing the silver electrode was polished first on an optical flat with a 600 grit silicon carbide (Buehler) slurry using a figure-of-eight motion, until the silver appeared flush with the Kel-F. It was then lapped on a Texmet pad with a 600 grit silicon carbide using a back-and-forth motion until the silver acquired a mirror finish. Next it was polished on a 2 7/8 in. diameter wheel spinning at 45 rpm, with a 0.5 µm alumina (Buehler) slurry using a V-motion. Finally the block was polished with 0.05 µm alumina on Microcloth (Buehler). The stainless-steel auxiliary electrode block was first polished with a 600 grit silicon carbide slurry, and finally with 1 µm diamond paste (Buehler) on Microcloth. Repolishing of the silver was required after about 100 injections, or about three times per week in continuous use. The reference electrode is located downstream of the working electrode in a 1 1/2 in. long x 1 1/8 in. diameter Nylon rod machined to produce a union-tee. To make connection to the electrochemical cell, one end of the tee was drawn turned down to 1/4 in. O.D. and threaded to screw into the 1/4-28 bottomed outlet port in the auxiliary electrode block. The other end of the tee was drilled and tapered to accept a 1/16 in. Swagelok bulkhead union. The 3 in. long x 5/8 in. diameter Nylon reference electrode housing screwed into the top of the Nylon tee through a central 3/8-24 tapped port. A calomel internal element (removed from a discarded calomel electrode)

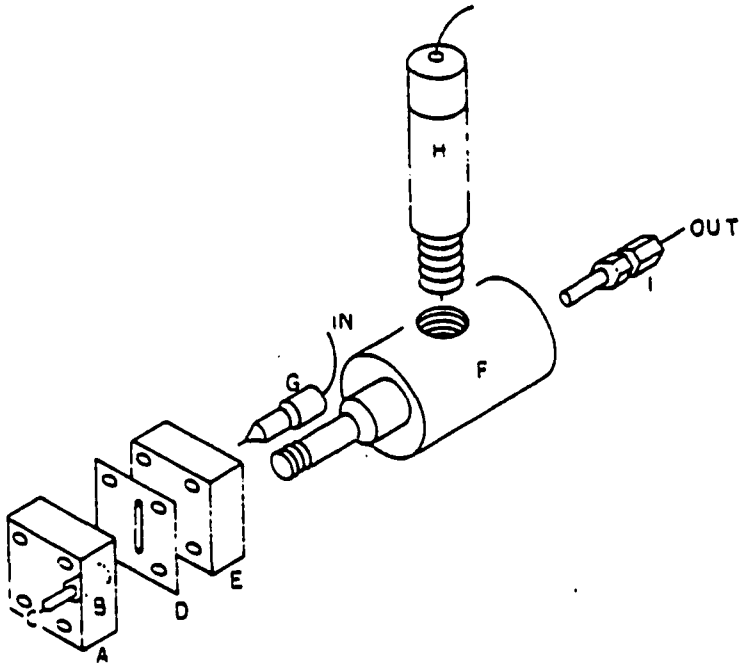

Fig. 1. Electrochemical detector cell. A = Working electrode block; B = silver electrode; C = pinjack connector; D = PTFE spacer; E = auxiliary electrode block; F = reference electrode cavity; G = inlet from HPLC column; H = calomel reference electrode housing; I = Swagelok bulkhead fitting outlet.

epoxied into a 3/8 in. O.D. flanged Delrin bushing which positioned the calomel electrode centrally in the Nylon housing. The liquid junction between the effluent stream and the calomel electrode was made with a 1/16 in. O.D. plug forced into a hole in the bottom of the Nylon housing. The plug was ground from pieces of a porous ceramic (unfired clay) cup used for electrochemical demonstrations. The calomel housing was filled with 3 M NaCl solution.

POTENTIOSTAT

A laboratory-built potentiostat based on a standard inverting configuration was used to control the potential applied to the auxiliary electrode. A circuit diagram is shown in Fig. 2. An electrometer based on an Analog Devices 52K module, shown in Fig. 3, was used to convert electrochemical current to voltage, which fed a Linear Instruments 1700 strip-chart recorder. In addition, a peak detector based on a circuit of Strohl and Curran (2) outputted the electrochemical peak current to a Keithley Model 179 DVM, to provide a value more accurate than that obtained from the recorder. The PC boards were mounted on the detector housing walls to minimize the distance between cell components and electronic controllers.

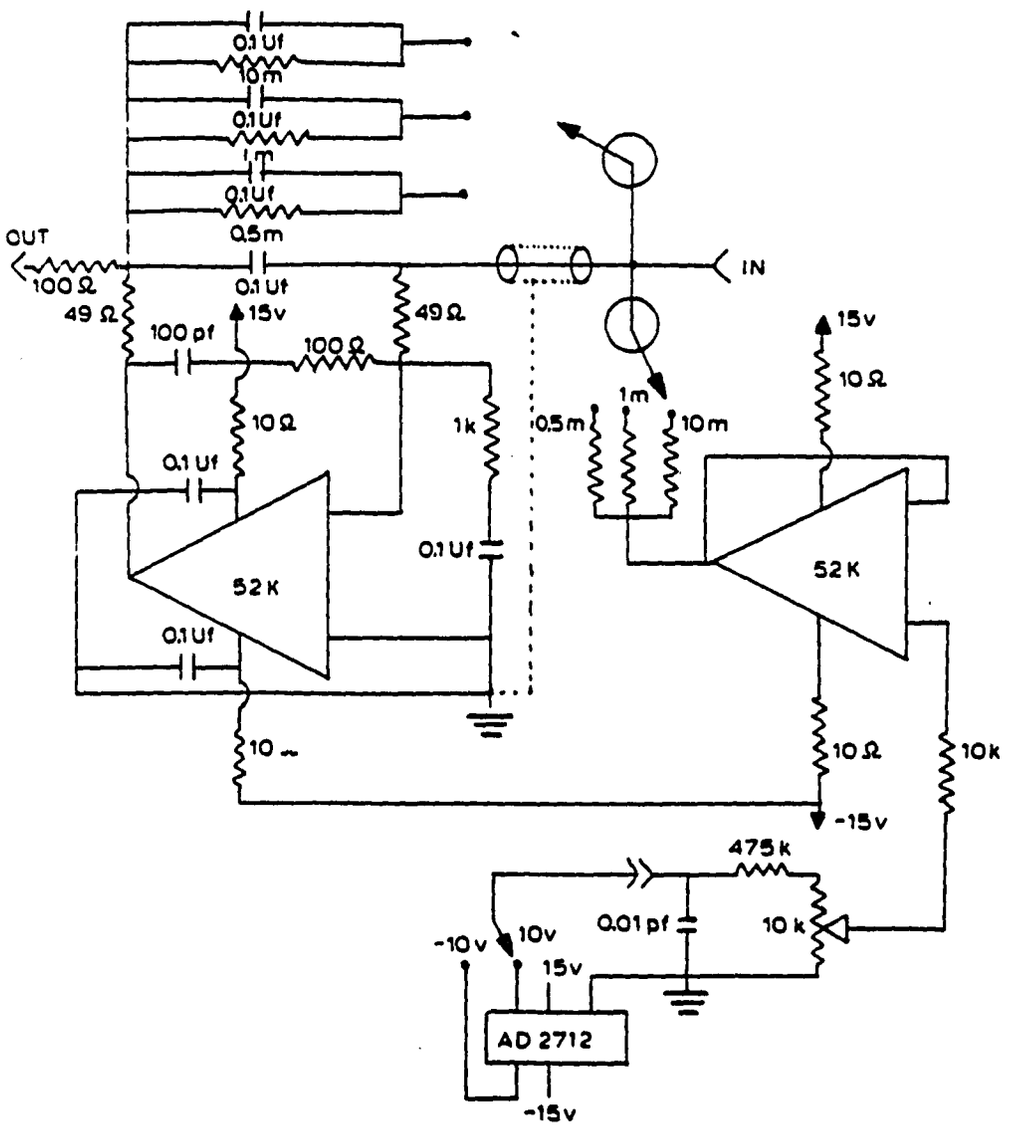

Figure 2. Circuit diagram of potentiostat.

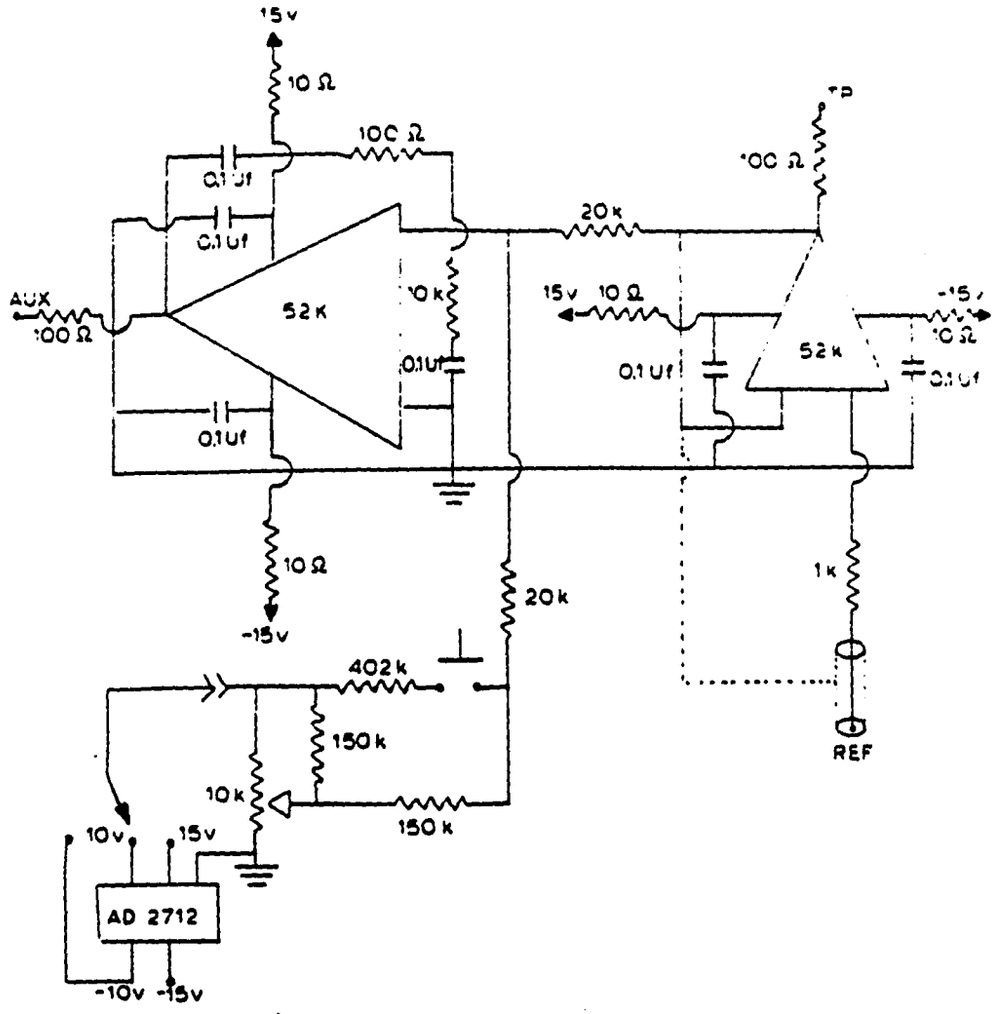

Figure 3. Circuit diagram of electrometer

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