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# EVALUATION OF PROTEOLYTICALLY RELEASED CARBOHYDRATE-CONTAINING PEPTIDES OF BOVINE PROTHROMBIN FRAGMENT 1 USING ELECTROSPRAY IONIZATION MASS SPECTROMETRY AND CAPILLARY ELECTROPHORESIS

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## ABSTRACT

Described here is a method used to determine the sialic acid content of the structurally distinct carbohydrates attached at N77 and N101 of bovine prothrombin fragment 1. The protein is proteolytically cleaved with α-chymotrypsin to release two glycopeptides, each containing one of the two glycosylation sites. Electrospray ionization mass spectrometry and capillary electrophoresis are used to determine the structural difference between the carbohydrates of each of these glycopeptides. N77 bears a carbohydrate containing as many as 4 sialic acid (*N*-acetylneuraminic acid) residues, whereas the carbohydrate attached at N101 may carry as many as 5 sialic acid residues.

Partially desialated forms of both of the native carbohydrate chains are observed. For both the N77 and N101-linked carbohydrates, the 1, 2, and 3-sialic acid-containing forms predominate. UV absorbance detection of the peptide-free, underivatized carbohydrate moieties is also demonstrated.

## INTRODUCTION

Glycoprotein activity has been implicated in the modulation of a wide range of functions in living organisms. Much of this functional diversity can be attributed to the carbohydrates attached to these biomolecules. Carbohydrates participate in both physicochemical and biological regulation. Their participation in physical phenomena includes the modification of protein charge, solubility, folding, conformational stability, and proteolytic accessibility.[1,2] In biological processes the carbohydrates of glycoproteins are known to regulate efficacy,[3] to serve as receptors for cellular interactions,[4] to modulate immunological responses,[5] and to function as recognition sites for targeting specific cellular species.[6] Furthermore, carbohydrates can play an important physiological role in the regulation of circulatory lifetimes of glycoproteins such as prothrombin.[7] The gradual biological removal of sialic acid (*N*-acetylneuraminic acid) residues, called desialation, occurs during the "molecular lifetime" of serum glycoproteins, giving rise to an appropriate molecular trafficking phenomenon: only the older, desialated glycoprotein molecules can interact with a hepatic receptor and be consequently removed from circulation for lysosomal degradation.[8] Sialic acids are also known to affect protein conformation and cell-cell adhesion.[7] One study demonstrates that enzymatic removal of sialic acids from blood coagulation protein factor IX results in diminished clotting efficiency.[9]

Attempts to elucidate the structures of glycoprotein carbohydrates are frequently hindered by both the chemical complexity and frequent absence of native chromophoric groups in free carbohydrates. Analysis can be further complicated by heterogeneity resulting from the existence of multiple protein glycoforms. Until the recent study of erythropoietin heterogeneity made by Rush and co-workers,[10] characterization of the carbohydrate portion of glycoproteins was typically performed after cleaving it from the protein backbone. The strategy presented here, which employs chymotryptic glycopeptides, uses very small sample sizes, requires minimal sample handling, and eliminates the need for derivatization.

Prothrombin is a blood coagulation zymogen present in blood serum at a concentration of about 100 μg/mL. Prothrombin, together with the other zymogens involved in the enzyme activation cascade of blood coagulation, contains several important post-translational modifications, among which are glycosylation, or carbohydrate attachment. The structures of three structurally distinct carbohydrate chains in bovine prothrombin were determined in the 1970s by chromatographic and electrophoretic analysis of digests produced by hydrazinolysis and exoglycosidase treatment.[11,12] Variation in maximum sialic acid content was established as one of the principal structural differences among these three carbohydrate chains of bovine prothrombin. The positions at which carbohydrates are attached to the protein backbone were later identified from amino acid sequencing; N-linkages to N77, N101 and N376 were determined.[13]

However, these studies did not indicate the specific sialic acid content of the carbohydrates attached at each of the three glycosylation sites. We sought to establish the sequence-specific sialic acid content for each of the carbohydrates of bovine prothrombin fragment 1 (bf1). Bf1 is the 156-residue amino-terminal portion of prothrombin, and hence includes the N77 and N101 glycosylation sites. We report the analysis of the variation in sialic acid content of the N77 and N101-linked carbohydrate moieties of bovine prothrombin.

The primary bioanalytical tools employed in the research described here include electrospray ionization mass spectrometry (ESI-MS) and capillary electrophoresis (CE). ESI-MS is a well-established method for the analysis of biomolecules.[14] ESI-MS provides the "soft" ionization needed for applications in which mass analysis of unfragmented biological macromolecules is desired, and is quite useful in characterizing the heterogeneity of glycoproteins.[15-17] A distribution of multiply-charged molecular ions is commonly observed in an ESI mass spectrum, which is generally deconvoluted to determine molecular weights. CE, the second analytical technique of importance to the research presented here, affords extremely efficient separations of tiny quantities of sample.[18,19]

Previous reports documenting the success of CE in studying carbohydrate structure have been reviewed by Novotny.[20] Successful CE analysis of carbohydrates often requires the use of charged complexing agents to effect separation, and generally also involves derivatization for spectroscopic detection. However, the mixtures analyzed here contain components that differ strictly in the number of negatively charged sialic acid residues they contain, and are readily separated without complexing or ion-pairing additives. Furthermore, no derivatization is required for the UV absorbance detection of these analytes.

## MATERIALS

Sialidase (neuraminidase, from *Clostridium perfringens*), α-chymotrypsin (TLCK treated, from bovine pancreas), trifluoroacetic acid (TFA), and tris(hydroxymethyl) aminomethane (tris) were obtained from Sigma Chemical Co. (St. Louis, MO). Periodic acid and *tert*-butyl alcohol were supplied by Aldrich Chemical Co. (Milwaukee, WI). Sodium chloride and cupric sulfate were purchased from Mallinckrodt (Paris, KY) and EM Science (Gibbstown, NJ), respectively. Sodium hydroxide pellets, hydrochloric acid, acetic acid, and resorcinol were obtained from Fisher Scientific (Pittsburgh, PA). N-glycosidase F (from *Flavobacterium meningosepticum*) was purchased under the trade name N-glycanase from Genzyme (Cambridge, MA). Bovine prothrombin was isolated from blood serum as described by Mann[21] and cleaved with *Echis carinatus* venom (Sigma) to generate bf1 as reported by Pollock et al.[22] Deionized water for the preparation of aqueous solutions was obtained from a Hydro (Research Triangle Park, NC) dual-cartridge purification system; ultra-high purity water, methanol, and acetonitrile for instrumental analyses were obtained from Fisher. All additional reagents used for N-terminal Edman degradation sequencing and amino acid analysis were supplied by Applied Biosystems (Foster City, CA).

## METHODS

### Glycopeptide Isolation

Chymotryptic digestion of 0.5 μmol bf1 (20 mM tris, 100 mM NaCl, 0.02% $NaN_3$, pH 7.4) was begun with a substrate-to-enzyme ratio of 50:1 (w/w). After 14 h of incubation at room temperature, the substrate-to-enzyme ratio was adjusted to 25:1, the temperature was increased to 25°C, and the proteolysis was allowed to proceed for an additional 2 h.

Chromatographic separation and preparatory collection of all proteolytically released peptides was performed on a Spectra Physics SP8800 HPLC (San Jose, CA) using a 10.0 x 250 mm, 5 μm particle size, $C_{18}$ reversed phase column (Rainin Microsorb) with a flow rate of 2.0 mL/min. Mobile phase A consisted of 0.1% (v/v) TFA in water and mobile phase B contained 0.1% TFA in acetonitrile. Chromatography was performed using a linear gradient of 0 to 70% mobile phase B over a total run time of 60 min. UV absorbance at 220 nm was monitored using a Spectra-Physics SP8450 on-line variable wavelength detector (San Jose, CA).

### Glycopeptide Identification

Chromatographed peptides bearing sialic acid-containing carbohydrates were identified using the periodate-resorcinol colorimetric determination described by Jourdian and coworkers.[23] A 0.1 mg portion of each peptide collected was used for the assay. Absorbances were determined using a Gilford model 260 spectrophotometer (Oberlin, OH).

### Glycopeptide Digestion

Sialic acids were removed from glycopeptides using 0.1-0.2 units of sialidase per 1.0 mg peptide (20 mM tris, 100 mM NaCl, 0.02% $NaN_3$, pH 6.5). Digests were typically performed at room temperature on 10-20 μg (about 2-4 nmol) of peptide. Aliquots removed during digestion were immediately analyzed by CE.

Glycopeptides were deglycosylated using 0.015 units of N-glycosidase F per 1.0 mg peptide (20 mM tris, 8 mM sodium phosphate, 105 mM NaCl, 5% glycerol, 0.02% $NaN_3$, pH 7.4). Digests were typically performed on a 10 μg sample of peptide. Following overnight incubation at 37°C, the samples were analyzed by CE.

### Amino Acid Sequencing and Amino Acid Analysis

All peptides studied were identified by N-terminal Edman degradation sequencing using an Applied Biosystems Sequenator model 475SA (Foster City, CA). After each cycle, the phenylthiohydantoin (PTH) amino acid derivatives were identified using reversed phase HPLC. Amino acid analysis was performed according to the manufacturer's instructions using an Applied Biosystems analyzer equipped with a Model 420A derivatizer, 120A Amino Acid PTH analyzer, and 920 Data Analysis Module.

### Electrospray Mass Spectrometry

Mass spectra were collected using a Vestec Model 201A quadrupole mass spectrometer (PerSeptive Biosystems/Vestec Mass Spectrometry Products, Houston, TX) equipped with an ESI source. All samples were prepared by dissolving lyophilized material in 1:1 (v/v) methanol/4% acetic acid to a concentration of approximately 1 mg/mL. Samples were continuously infused into the ESI source at 2 to 4 μL/min using a syringe pump (Model 341B, Sage

Pumps, Boston, MA). Spectrometer operating conditions were as follows: ES needle voltage, 1.7-2.0 kV; nozzle potential, 276 V; repeller potential, 20 V; ES probe temperature, 46-58 ± 5°C; block temperature, ≈ 259°C; lens temperature, ≈ 116°C. Needle-to-nozzle and nozzle-to-skimmer distances were adjusted as necessary to obtain sufficient signals. Electrospray mass spectra (1000-2000 Da) were collected in SIM mode with a Vector/2 data system (Teknivent Corp., Maryland Heights, MO). Multiple ESI-MS scans were averaged to improve signal-to-noise ratios.

### Capillary Electrophoresis

Electrophoresis instrumentation was constructed as described by Jorgenson and Lukacs[18] using a Spellman (Plainview, NY) high voltage power supply. Separations were conducted at room temperature with a 100 cm (75 cm effective length), 50 μm i.d., 360 μm o.d., unmodified fused silica, polyimide-coated capillary (Polymicro Technologies, Phoenix, AZ). A run buffer containing 50 mM NaCl and 10 mM tris (pH 8.4) was employed with a separation voltage of 22 kV. Samples were introduced at the anode end of the capillary using the electromigration method (12 kV for 5 sec). Detection was accomplished with a Linear Model UVIS 200 on-capillary spectrophotometric detector (Linear, Reno, NE). Electropherograms were acquired using an Apple Macintosh computer with a Rainin MacIntegrator (Ridgefield, NJ) data collection package. Data was filtered with a 5-point binomial filter[24] using the Igor Pro software package (WaveMetrics, Lake Oswego, OR).

## RESULTS AND DISCUSSION

### ESI-MS of Bf1

ESI-MS analysis of native bf1 reveals several populations of protein species that differ in weight by multiples of 300-330 Da (Figure 1). These differences are attributed to variation in the number of sialic acid residues associated with the carbohydrate. To ensure that no significant artifactual desialation of bf1 occurred during the HPLC separations (mobile phase pH ≈ 3) performed in these studies, colorimetric determinations of sialic acid content were performed on both chromatographed and unchromatographed protein (data not shown). No difference in sialic acid content was apparent between the two samples.

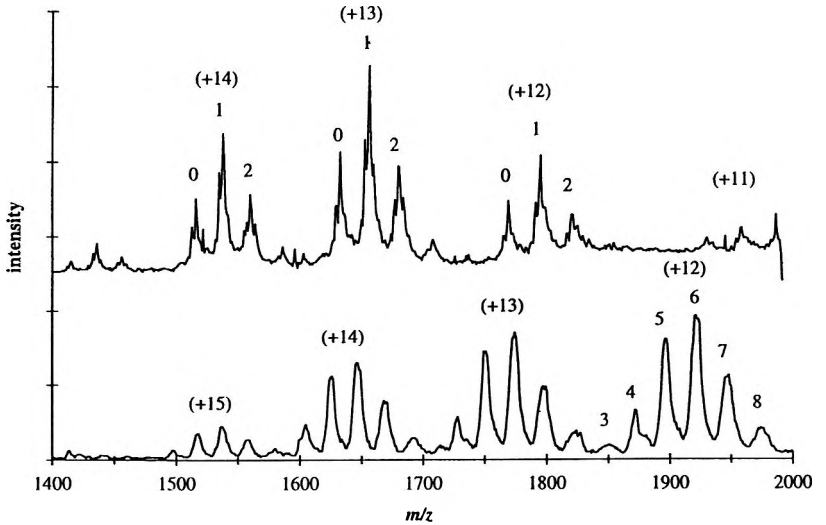


**Figure 1**. ESI mass spectra of native (lower trace) and sialidase-treated (upper trace) bf1. The number of sialic acids contained by each species is indicated; the charge *z* on ions in each group is indicated in parenthesis. See Table 1 for the molecular weights obtained by computer deconvolution of the spectrum. Details of sample preparation and mass spectrometer operating conditions are presented in the Methods section.

**Table 1**

**Glycoforms for Native and Desialated Bf1 Detected by ESI-MS**

| No. of Sialic Acids | Charge Species | MW (From ESI-MS) | MW (Calculated)[a] |
|---|---|---|---|
| 0 | +14, +13, +12, +11 | 21,240.32 (±0.94) | 21,344.48 |
| 1 | +14, +13, +12, +11 | 21,547.21 (±2.68) | 21,619.74 |
| 2 | +14, +13, +12, +11 | 21,853.55 (±1.08) | 21,895.00 |
| 3 | Barely Detectable | Not Calculated | 22,170.26 |
| 4 | +15, +14, +13, +12 | 22,453.50(±1.88) | 22,445.51 |
| 5 | +15, +14, +13, +12 | 22,749.76 (±2.72) | 22,720.77 |
| 6 | +15, +14, +13, +12 | 23,042.72 (±2.60) | 22,996.03 |
| 7 | +15, +14, +13, +12 | 23,355.37 (±10.75) | 23,271.29 |
| 8 | +15, +14, +13, +12 | 23,686.62 (±4.49) | 23,546.55 |

[a]Theoretical molecular weight computed using the amino acid sequence and carbohydrate composition of the assigned bf1 glycoform.

A mass spectrum was also acquired after treatment of bf1 with sialidase, an enzyme which cleaves sialic acids from glycoproteins. Examination of the untreated and sialidase-treated bf1 spectra shown in Figure 1 reveals a total of nine major peaks (species containing 0 through 8 sialic acids), corresponding to a maximum content of 8 sialic acid residues. As seen in Figure 1, the relative intensities for the bf1 peak groups increase with *m/z*, indicating that the predominant charge species may lie above the *m/z* range limit (2000) of the mass spectrometer. However, deconvolution and subsequent molecular weight determinations can be made based on the signals from molecular ions within the *m/z* range of the instrument. The molecular weights obtained by deconvolution of the mass spectra are presented in Table 1, along with the theoretical molecular weight of each species. Differences in the theoretical and experimental weights are attributed to additional microheterogeneity of the protein (see below).

## General Strategy

Peptides are generated by proteolysis of bf1 with α-chymotrypsin and are isolated using semipreparative HPLC. Glycopeptides are distinguished from non-glycosylated peptides using a periodate-resorcinol colorimetric determination.[23] Amino acid sequencing and amino acid analysis are used to determine the amino acid sequence of each glycopeptide. Given this sequence and the common core composition of the carbohydrate chains, a theoretical molecular weight, which does not account for sialic acid residues, is calculated for each glycopeptide. The number of sialic acids contained by each glycoform is then determined by ESI-MS analysis of the glycopeptides. A complimentary series of experiments in which the isolated glycopeptides are analyzed by CE before and after treatment with several enzymes is also conducted. The ESI-MS and CE profiles of the sialic acid content are then used to establish the maximum number of sialic acid residues contained by each carbohydrate.

## Isolation and Identification of Chymotryptic Glycopeptides

Reversed phase semi-preparatory HPLC was used to isolate each glycopeptide from an α-chymotryptic digest of bf1. Peptides were collected preparatively, separated into aliquots of 0.15-0.3 mg each, and lyophilized. The two glycopeptides each elute as singlet peaks despite the differences in sialic acid content of their carbohydrate moieties. This is demonstrated by the fact that only three sialic acid-containing bands, corresponding to the two glycopeptides and a small amount of undigested protein, were detected during the entire HPLC run. Amino acid analysis and N-terminal Edman degradation

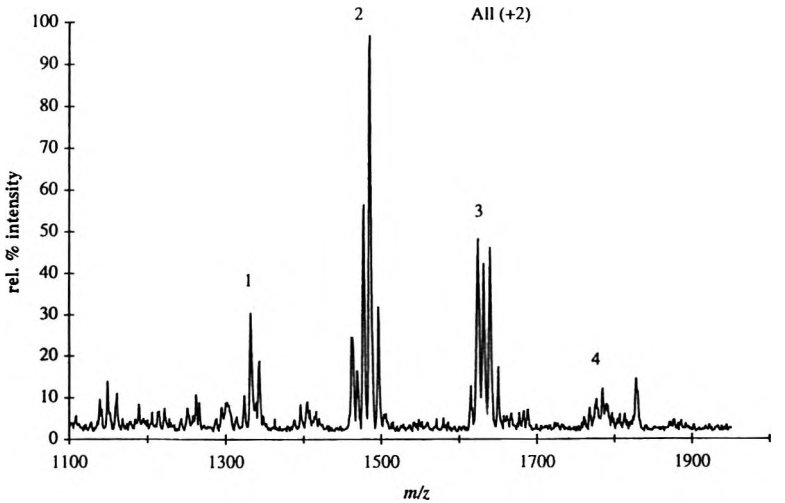


**Figure 2**. ESI-MS of the N77 glycopeptide of bf1. The number of sialic acids present on each species is indicated; all ions have a charge state $z$ of +2. Table 2 shows the estimated weights of glycoforms obtained after deconvolution of this spectrum. See Methods section for experimental conditions.

## Table 2

### Glycoforms for N77 Glycopeptide of Bf1 Detected by ESI-MS

| No. of Sialic Acids | Charge Species | MW (From ESI-MS)[a] | MW (Calculated)[b] |
|---|---|---|---|
| 1 | +2 ($m/z \approx 1340$) | 2680 | 2,654.62 |
| 2 | +2 ($m/z \approx 1480$) | 2960 | 2,945.88 |
| 3 | +2 ($m/z \approx 1630$) | 3260 | 3,226.06 |
| 4 (minor) | +2 ($m/z \approx 1780$) | 3560 | 3,516.30 |

[a]Peak locations determined by Vector/2 centroid peak finder deconvolution software.

[b]Theoretical molecular weight computed from the known amino acid sequence and carbohydrate composition of the assigned structure.

sequencing were used to determine the primary amino acid sequence of each of the glycopeptides. R75-W81 bears the N77-linked carbohydrate, and R91-F114 bears the N101-linked carbohydrate. It is worthwhile to note that α-chymotrypsin is not expected to catalyze cleavage on the C-terminal side of W90 to generate the observed N101 glycopeptide; however, amino acid analysis, sequencing, and mass spectrometric analysis all support the assignment of the structure reported here. Sequencing and CE analyses also demonstrated the presence of a contaminating tripeptide, A43-Y45, in the N77 glycopeptide fraction. The removal of this contaminant was effected by washing with three volumes of water in a Centricon-3 (3000 Da cutoff; Amicon, Beverly, MA) and was monitored using CE.

## ESI-MS Analysis of Glycopeptides

The deconvoluted mass spectrum of the N77 glycopeptide (Figure 2; Table 2) shows three major peaks corresponding to glycoforms containing 1, 2, and 3 sialic acids. This spectrum was generated using the signal from the only detectable charge state, $z = +2$. The molecular weight corresponding to the heaviest of the major species in this spectrum, 3260 Da, agrees well (within about 1%) with the calculated theoretical molecular weight of 3226 Da for the 3-sialic acid-containing glycopeptide. An extremely low signal from a species of molecular weight 3560 Da suggests that a glycoform containing 4 sialic acids is also present.

Glycoforms containing zero to four sialic acids are observed in the mass spectrum of the N101 glycopeptide (Figure 3; Table 3). The deconvoluted mass spectrum was obtained using signals from the ionized species in the three charge states ($z = +3, +4, +5$) that fall within the $m/z$ range of the spectrometer. The theoretical molecular weight of the 4-sialic acid-containing glycopeptide is 5607.57 Da, in excellent agreement (within 1%) with the experimentally observed value of 5643.51 Da for the heaviest glycoform. The consistent incremental molecular weight difference of approximately 300 Da, corresponding to one sialic acid residue, can be seen in Tables 2 and 3. Examination of individual "peaks" in both mass spectra (Figures 2 and 3) reveals fine structure that results from additional structural microheterogeneity of the protein. Such heterogeneity could be attributed, for example, to a variety of substitutions to which sialic acids are subject, including hydroxymethylation and hydroxyesterification.[7] Note that the 5-sialic acid-containing species observed by CE (see below) is not readily identified in the mass spectrum, probably owed to insufficient signal.

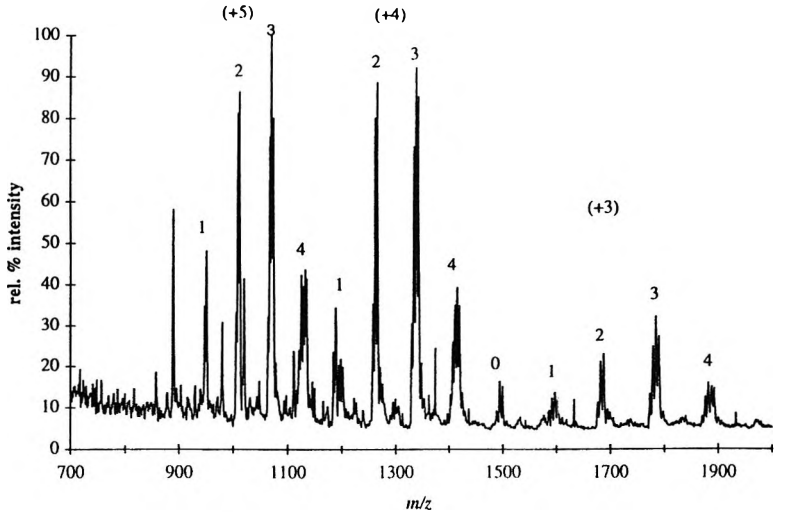


**Figure 3**. ESI-MS of the N101 glycopeptide of bf1. Peak numbers indicate the number of attached sialic acids, and the charge *z* for each group of species is indicated in parenthesis. Molecular weights obtained from the deconvoluted mass spectrum are shown in Table 3. Additional experimental conditions are given in the Methods section.

**Table 3**

**Glycoforms for N101 Glycopeptide of Bf1 Detected by ESI-MS**

| No. of Sialic Acids | Charge Species | MW (From ESI-MS)[a] | MW (Calculated)[b] |
|---|---|---|---|
| 0 | +3 | Not Calculated | 4,506.54 |
| 1 | +5, +4, +3 | 4,745.63 (±0.34) | 4,781.79 |
| 2 | +5, +4, +3 | 5,047.08 (±0.14) | 5,057.05 |
| 3 | +5, +4, +3 | 5,354.10 (±8.49) | 5,332.31 |
| 4 | +5, +4, +3 | 5,643.51 (±4.66) | 5,607.57 |

[a]The molecular weight of the most abundant species in each peak group is reported.

[b]Theoretical molecular weight computed from the known amino acid sequence and carbohydrate composition of the assigned structure.

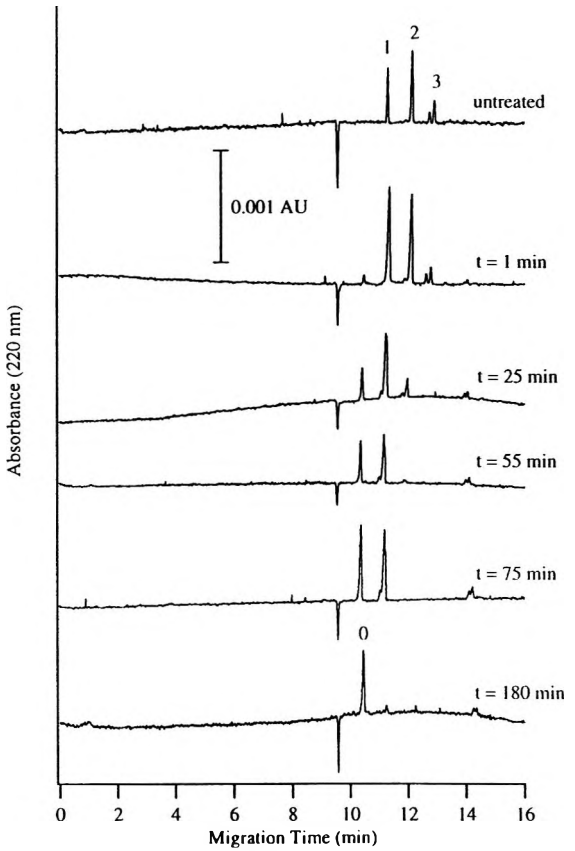


**Figure 4.** Electropherograms obtained during sialidase treatment of the N77 glycopeptide after the indicated incubation time. The number of sialic acids contained by each glycoform is indicated. Sample preparation and instrumental conditions are included in the Methods section.

## CE Analysis of Glycopeptides

Multiple peaks were observed in CE electropherograms of both glycopeptides (Figures 4 and 5), representing variation in the number of sialic acid residues contained by the carbohydrate chains. The strong dependence of electrophoretic mobility on sialic acid content is well-established and occurs as a result of the negative charge character of the sialic acid residue at moderate to high pH (CE run buffer, pH 8.4).

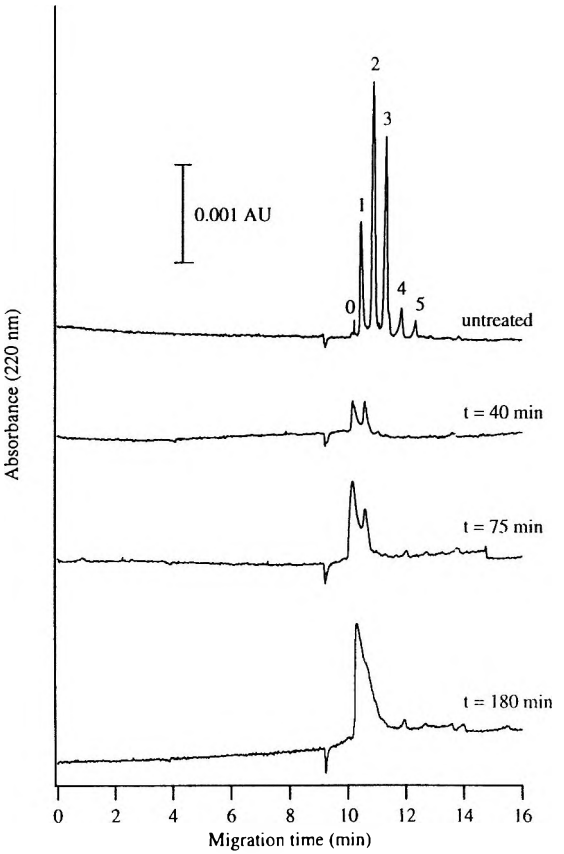


**Figure 5**. Electropherograms obtained during sialidase-catalyzed desialation of the N101 glycopeptide. CE runs were performed at the end of the incubation period indicated on each trace, as described in the Methods section. The number of sialic acids contained by each glycoform is indicated.

To provide further evidence that the observed heterogeneity arises from a distribution of sialic acid-containing species, enzymatic digestions of both glycopeptides were conducted and analyzed by CE. These experiments employed sialidase and N-glycosidase F, which catalyze sialic acid removal and deglycosylation respectively.

The electropherogram of the undigested N77 glycopeptide (Figure 4) contains three peaks, representing the species containing 1, 2, and 3 sialic acids. Adjacent peaks represent glycoforms that differ in composition by one negatively charged sialic acid residue, with the population containing three sialic acids possessing the longest migration time (12.8 min). The presence of

approximately 5% glycerol, added to increase enzyme stability, is responsible for a brief baseline drop that marks the electroosmotic flow (EOF). Specific peak assignments are substantiated by the data acquired during the course of sialidase digestion as shown in Figure 4. The gradual emergence of a fully desialated (0-sialic acid-containing) glycoform is observed; exhaustive treatment (180 min) converts the entire population to this species. Minor peaks (e. g., unlabeled peak at 12.7 min) are likely due to further heterogeneity of the protein which may include isomeric variation. CE data indicates that the N77 carbohydrate bears a maximum of 3 sialic acids. The 4-sialic acid-containing population, identified as a minor component by mass spectrometry, was not detected by CE.

In the electropherogram of the N101 glycopeptide (Figure 5), glycoforms containing zero to five sialic acids are observed. The 1, 2, and 3-sialic acid containing species are clearly the predominate forms, as indicated by their larger peak areas. The gradual disappearance of the sialated variants during digestion with sialidase supports these peak assignments. All glycoforms are ultimately desialated to generate a 0-sialic acid-containing population, which has a migration time of 11 min.

The peak shapes observed in the sialidase-treated samples are irregular; the source of this unexpected behavior, which is also observed after N-glycosidase F treatment (see below) of the N101 glycopeptide, is presently unknown. One potential cause would be a peak tailing effect originating from the increased analyte/capillary wall interaction that could be exaggerated by removal of negatively charged moieties from this large glycopeptide.

In a separate set of experiments, each glycopeptide was exhaustively incubated with N-glycosidase F, which effects cleavage of the *N*-linked carbohydrate from the peptide backbone, leaving an aspartic acid residue. The CE electropherogram of each of these digests (Figure 6) shows a peak corresponding to the deglycosylated peptide as well as multiple peaks corresponding to populations of free carbohydrate glycoforms. The free carbohydrate peaks are barely detected, but exhibit an absorbance at 220 nm, apparently because of several UV-active amide bonds.

The data presented here show that the carbohydrate of bovine prothrombin linked to N77 bears a maximum of 4 sialic acids, and the carbohydrate moiety attached at N101 may contain as many as 5 sialic acids. The most abundant forms of both the N77 and N101 carbohydrates are those containing 1, 2, or 3 sialic acid residues.

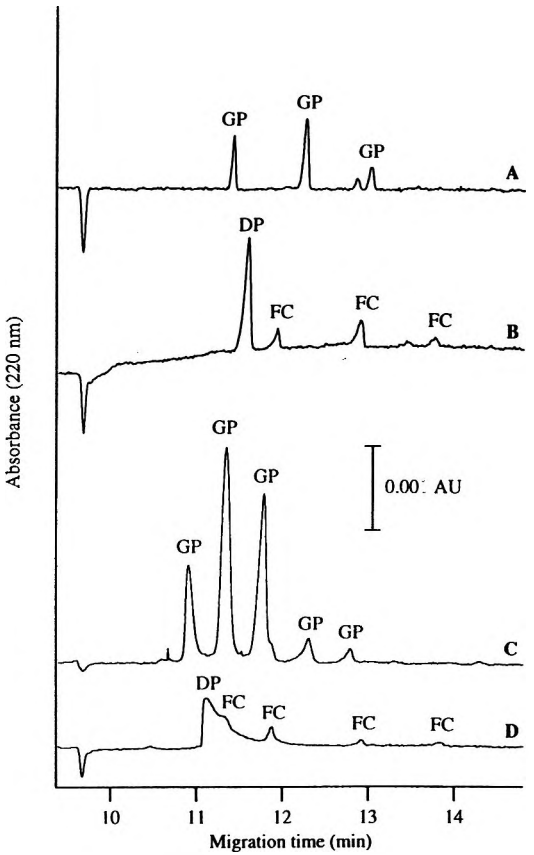


**Figure 6**. Electropherograms obtained before and after N-glycosidase F-catalyzed removal of the carbohydrate chains of both glycopeptides. A and B show N77 before and after N-glycosidase F treatment respectively; C and D show N101 before and after treatment respectively. Peaks are labeled accordingly: GP, glycopeptide; DP, deglycosylated peptide; FC, free carbohydrate. Additional experimental conditions are given in the Methods section.

Evaluation of structural variation in the carbohydrates of serum proteins such as prothrombin could greatly speed diagnosis and ultimately be used to improve treatment strategies for coagulation disorders thought to be related to glycosylation.[25-28] Many of the coagulation proteins are, in fact, similar to prothrombin in structure[29,30] and could be effectively studied using methods developed for the characterization of prothrombin. Analytical schemes employing sample-conservative techniques such as ESI-MS and CE are especially attractive for projects aimed at the characterization of other coagulation proteins, which are present in blood serum at concentrations as low

as 1/200th of that of prothrombin. The approach set fourth here should facilitate identification of the carbohydrate moieties attached at specific glycosylation sites in proteins.

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# RETENTION OF ACIDIC AROMATIC COMPOUNDS IN ION EXCLUSION CHROMATOGRAPHIC SEPARATIONS

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## ABSTRACT

Aromatic acidic compounds have been separated by ion exclusion chromatography. The theoretical equation, which relates the retention volume of an eluted acid to its dissociation constant, failed for aromatics, which show higher retention volumes than predicted. An important role is played by hydrophobic adsorption, which can be increased by addition of ion interaction reagents to the mobile phase .

## INTRODUCTION

Ion exclusion chromatography (IEC) is a widely applied technique to separate ionic compounds, since the retention of a single compound depends on the ratio of concentrations of its ionised and neutral forms.[1] The characteristic feature of IEC technique is the electric charge of dissociated ion-exchange resin functional groups which has the same sign of the electric charge of the analysed

ionic compound. It follows that samples containing negatively charged ions, as dissociated acidic compounds, are separated on cation exchange resins with anionic functional groups. For this purpose a large ion exchange capacity column is preferred, which can be obtained maximising the column dimensions and the functional group concentration in the support, and using strong ion-exchange resins.

When such a column is filled up with water, which is pumped through it as a mobile phase, the water molecules build up hydration spheres around the dissociated functional groups of the support. Water contained in the pores of the support and in the hydration spheres is immobilised and it forms the stationary phase, in which the resin functional group ions and the hydrogen ions are dissolved. The retention mechanism of ion exclusion[2] is based on the following phenomenon. Neutral, uncharged molecules are allowed to penetrate the resin, while similarly charged coions are repulsed owing to the presence of dissociated functional groups immobilised in the stationary phase. The hydrated resin network behaves like a semipermeable membrane between the stationary phase and the mobile phase. With the exception of the covalently bound functional groups and of the counterions because of the electroneutrality requirement, all other species are freely exchanged through such a hypothetical membrane, which is permeable to neutral molecules. As a consequence, strong acids, which are completely dissociated and, therefore, electrostatically repulsed are eluted, not separated, in the column dead volume ($V_M$), which corresponds to the volume of the mobile phase in the column. On the other hand, undissociated compounds can completely penetrate the resin and are also not separated being eluted in a volume correspondent to the sum of the inner and the dead volumes of the column, where the inner column volume is just the volume of its stationary phase. This behaviour makes the determination of the inner and dead column volumes straightforward as it has been confirmed by Tanaka et al.[3] Only the acids of intermediate strength, with their dissociation constants falling in the range $10^{-7}$ - $10^{-2}$, can be separated by this technique.

Ion exclusion can seldom be considered as the sole retention mechanism even on an ion-exclusion resin. Like, in other chromatographic techniques, it is classified according to the primary mechanism of solute retention. This primary mechanism in IEC is coulombic repulsion between solute and dissociated groups of the resin. Besides ion exclusion, let us recall hydrophobic adsorption on the resin network as in reversed phase chromatography, size exclusion,[4,5] effect of functional group screening in the analysed sample, normal phase retention, van der Waals, and polar interactions of the sample compound with the support.[1]

The aim of the presented paper is to investigate how hydrophobic adsorption influences the retention of aromatic acidic compounds in ion exclusion chromatographic separations. To this purpose, the retention volumes of aliphatic and aromatic acids are compared with a theoretical model. Furthermore, the addition of ion interaction reagents is studied in order to investigate the possibility to govern hydrophobic adsorption.

## EXPERIMENTAL

### Materials

Analytical reagent-grade sulphuric acid (Carlo Erba, Milan, Italy) dissolved in triply distilled water at the selected concentration was used as mobile phase. Tetrabutylamonium bromides used as ion-interaction reagents were Aldrich-Chemie (Steinheim, Germany) products. All other chemicals were Research Grade products and were used without further purification.

### Apparatus

The chromatographic analyses were carried out by Beckman equipment consisting of a 110B Solvent Delivery Module, a 340 Organizer, and a 160 Absorbance Detector, and equipped with a HPX-72-O Bio-Rad (Richmond, USA) column (300 x 7.8 mm I.D., hydroxide form strong ion exclusion column packed with 11 μm particles of polystyrene-divinylbenzene copolymer, crosslinking 8%). The retention times of the eluted compounds were measured by a Hewlett Packard HP 3394A Integrator.

### Procedure

Triply distilled water was passed through a Millipore (Bedford, USA) Milli-Q water purification system, filtered through a Millipore 0.45 μm membrane filter, and degassed in an ultrasonic bath, before the addition of sulphuric acid. The column was equilibrated for at least 1 hour prior to being used.

All of the analyses were carried out at ambient temperature. Each sample was injected six times and the average value of its retention time was taken.

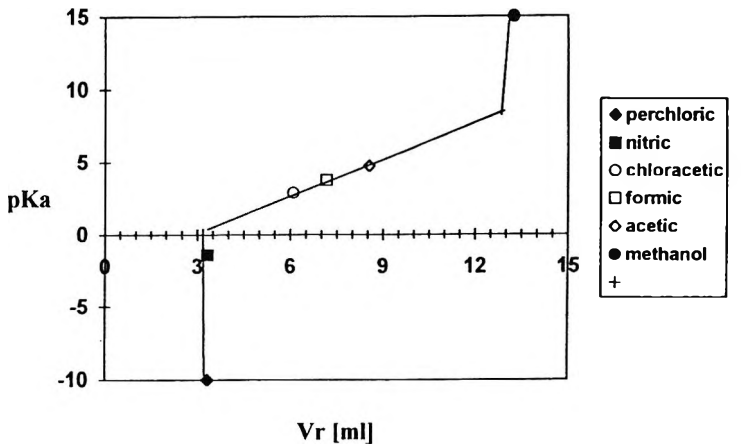


**Figure 1**. Effect of $pK_a$ values on retention volumes, $V_R$. Bic-Rad Aminex ion-exclusion HPX-87H organic acid analysis column, 300 x 7.8 mm I.D., hydrogen form 8% crosslinked cation exchanger, 9 μm particle diameter. Mobile phase: 1 mN $H_2SO_4$. Solute acids separated by pure ion-exclusion effect.

## RESULTS AND DISCUSSION

Many models have been used to describe the IEC mechanism. The simplest one, which assumes pure water as a mobile phase, equal inner and dead volumes, and the support functional groups completely dissociated, leads to simple equations.[6] The distribution coefficient, $K_d$, can be obtained from the chromatographic data:

$$K_d = (V_R - V_M)/V_S \qquad (1)$$

where $V_R$ and $V_S$ are the retention and the stationary phase volumes, respectively.

The influence of the dissociation constant of an acid on its retention volume is shown in Figure 1. The characteristic for the ion-exclusion s-shape dependence between the $pK_a$ and retention volume can be observed.

Strong acids, like perchloric or nitric, are eluted at the dead column volume ($K_d = 0$). Very weak acidic compounds are eluted at a distribution coefficient equal one. Other acids (chloroacetic, formic, acetic) are eluted between these boundaries, as confirmation of the theoretical predictions.[1,3]

**Table 1**

**Observed Retention Volumes (mL) Against Expected Retention Volumes from pKa Values**

| Acid | pKa | Vr obs. | Vr exp. |
|---|---|---|---|
| Benzoic | 4.20 | 72.00 | 7.4 - 8.2 |
| Salicylic | 3.00 | 44.87 | 6.0 - 6.8 |
| Anisic | 4.47 | 41.09 | 7.6-8.4 |
| Tannic | 4.32 | 20.03 | 7.5 - 8.3 |

From the s-shape of Fig. 1. the retention of other acids can be calculated. In Table 1 the found retention volumes of aromatic acids are compared with the calculated values. It can be observed that these compounds are characterised by much higher retention than expected on the basis of a pure ion-exclusion. It means that their retention is governed by the mixed ion-exclusion - hydrophobic adsorption mechanism.[5]

A theoretical model can be proposed.

The distribution coefficient ($K_d$) in the case of acidic solutes can be written as:

$$K_d = \frac{[HR]_S + [R^-]_S}{[HR]_M + [R^-]_M} \qquad (2)$$

where the subscripts M and S refer to the mobile and the stationary phase, respectively.

Under the conditions that a strong acid is used as a buffer and that its concentration is much higher than that one of solute, Eq. (2) can be written as:

$$K_d = \frac{[HR]_S + [HR]_S \, K_a / c_f}{[HR]_M + [HR]_M \, K_a / c_b} \qquad (3)$$

where $c_f$ and $c_h$ are the functional groups and the buffer concentrations, respectively, and $K_a$ is the dissociation constant of the acid.

**Table 2**

**Comparision of Retention Volumes (mL) and $pK_a$ Values of Some Isomers of Substituted Benzoic Acids. Mobile Phase: 1mN $H_2SO_4$ Column: Aminex HPX-87H**

| Acid | Ortho- | | Para- | | Meta- | |
|---|---|---|---|---|---|---|
| | $pK_a$ | $V_R$ | $pK_a$ | $V_R$ | $pK_a$ | $V_R$ |
| Toluic | 3.91 | 59 | 4.36 | 61.6 | 4.27 | 103. |
| Nitrobenzoic | 2.17 | 9.6 | 3.44 | 55.0 | 3.49 | 88.0 |
| Phthalic | 2.98 | 11.8 | 3.51 | 45.3 | 3.54 | 4.5 |

In an analytical chromatographic process we assume linear adsorption:

$$K_d = \frac{K_H\left(1+\frac{K_a}{c_f}\right)}{1+\frac{K_a}{c_b}} \qquad (4)$$

where $K_H$ consists of the two effects: the ion-exclusion and hydrophobic adsorption of the neutral molecule.

In the absence of adsorption the concentrations of the neutral molecules in the both phases are equal. Since in ion-exclusion columns the concentration of the functional groups is generally much higher than that of the solute, the second term of the numerator can be generally omitted.

From Table 1 it can be read out that the aromatic compounds are characterised by a very high retention. This is probably due to the interaction between their π-electron and the resin network. This effect was found even stronger for aromatic bases[1,4] whose retention was a hundred times higher than that expected from the pure ion-exclusion effect.

As reported in Table 2, substituted aromatic acids are eluted mainly in the order of ortho-, para-, meta-. Even if they show different values of their dissociation constants, such differences seem to be too small to explain the big changes in their retention. An explanation can be found in their different molecular volumes, which are related to their surface area. It can be observed that their retention is inversely proportional to their density.

**Table 3**

**Retention Volumes (mL) of Isomeric Nitrobenoic Acids in Different Mobile Phases (Bio-Rad Aminex Ion-Exclusion HPX-87H Organic Acid Analysis Column, 300 x 7.8 mm I.D., Hydrogen Form 8% Cross-Linked Cation Exchanger, 9 μm Particle Diameter)**

| | | | Mobile Phase | | |
|---|---|---|---|---|---|
| **Isomer** | **$pK_a$** | **ρ [g/mL]** | **Water** | **1mN $H_2SO_4$** | **0.5mM TBABr** |
| Ortho | 2.17 | 1.575 | 5.05 | 9.6 | 8.33 |
| Para | 3.44 | 1.55 | 5.14 | 55 | 49.05 |
| Meta | 3.49 | 1.494 | 9.3 | 88 | 74.49 |

As an example, m-nitrobenzoic acid is the last isomer eluted in the different experimental conditions, reported in Table 3. The meta- isomer is characterised by the smallest density value, 1.494 g/mL, in comparison with para- (1.55 g/mL) and ortho- (1.575 g/mL) isomers.

Since hydrophobic adsorption seems to play an important role in ion exclusion chromatographic separations, it seems useful to investigate how to govern it.

Hydrophobic adsorption can be decreased by addition of an organic modifier to the mobile phase. As a matter of fact,[7] it has been shown that cyclodextrine added to the mobile phase could also decrease retention.

On the other side, an increase of the hydrophobic adsorption can be obtained by addition of an ion-interaction reagent (IIR) to the mobile phase. In other chromatographic techniques,[8] it was found that the addition of small concentration of IIR to the mobile phase in the absence of buffer not only improved the retention, but led to more symmetrical peaks and avoided the dependence of the solute retention on its concentration. Also in IEC, IIR can be added to the mobile phase.

Since the electrostatic interactions between solutes and functional groups are very quick, there should not be time wasted waiting for column stabilisation. To confirm such a hypothesis, we changed the mobile phase from water to 0.5 mM tetrabutylamonium bromide (TBABr). The equilibrium was reached after few minutes. The same effect was found for the opposite variation.

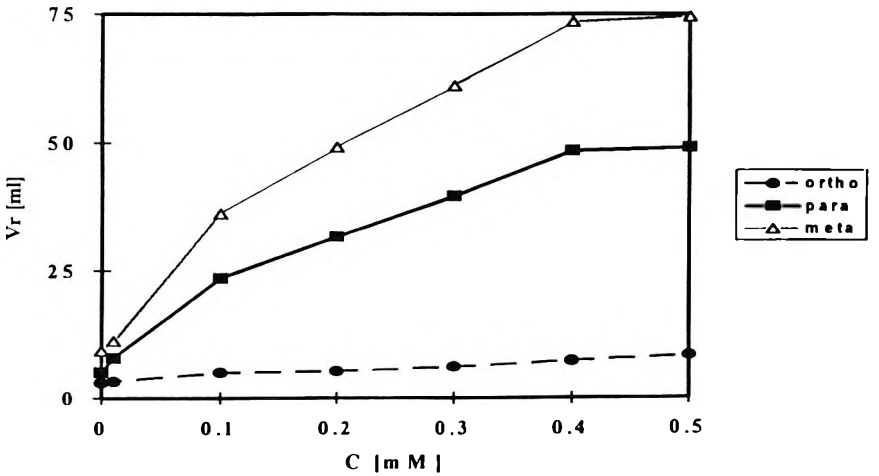


**Figure 2.** Influence of the tetrabutylammonium bromide concentration on the nitrobenzoic acids retention. Chromatographic conditions as in Fig. 1.

The influence of the IIR concentration on the retention for the isomeric nitrobenzoic acids is shown in Fig. 2. It can be seen that the addition of a small concentration of TBABr improves the separation of the isomers.

## CONCLUSIONS

Aromatic acidic compounds are characterised by higher retention than predicted by a pure ion exclusion mechanism. We suggested that the reason can be found in a hydrophobic adsorption on the resin network, which is constituted mainly of styrene and divinylbenzene copolymerised resins. It means that, in this case, the retention is governed by the mixed ion-exclusion - hydrophobic adsorption mechanism.

This adsorption can be increased by the addition of an ionic interaction reagent to the mobile phase. Since, for this purpose, a small concentration (0.01 mM) of ionic interaction reagent can be used in the absence of buffer, the overall result is an improvement of solute retention without influencing the detection system.

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# ADVANTAGES OF EVAPORATIVE LIGHT SCATTERING DETECTION FOR THE PURITY CONTROL OF COMMERCIAL CYCLODEXTRINS

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## ABSTRACT

Using an Evaporative Light Scattering Detector (ELSD) which is a universal detection system compatible with gradient elution, allowed satisfactory analysis of the non volatile impurities in cyclodextrins. ELSD performances for cyclodextrin analysis were evaluated. The detection capabilities of ELSD are better than those of RI and Polarimetric detectors (detection thresholds obtained with the ELSD are about 80 ng). Analyses were performed on different Phenyl columns with acetonitrile-water mobile phases in isocratic or gradient mode. Potential impurities of β-CD and of TM-β-CD, which included 7 linear (glucose to maltoheptaose), 2 unbranched cyclic (α- and γ-CD) glucose polymers and partially methylated cyclodextrins, have been determined.

As the ELSD response factors of these homologous compounds are highly similar, it is possible to evaluate impurity levels without previous identification. The methods presented provide good selectivity and sensitivity and can also be used to compare different sources of cyclodextrins.

## INTRODUCTION

β-cyclodextrin (β-CD) and methylated β-cyclodextrins are used in various fields, such as in the manufacture of cosmetics, food technology, the pharmaceutical industry and in the production of agrochemicals, for their ability to include different sized guest molecules.[1] However, the analysis as well as the isolation in high purity of these cyclodextrins are still a real problem.

Commercially, β-CD is produced from the enzymatic digestion of starch.[2] The β-CD is selectively purified from the crude digest containing compounds with highly different polarities like glucose, linear glucose polymers, cyclic hexamer (α-CD), cyclic octamer (γ-CD) and cyclic heptamer (β-CD). An analytical method was needed to determine the level of potential linear and unbranched cyclic polymeric glucose impurities in β-CD.

β-CD has often been modified chemically. Methylation of β-CD to form 2, 6 or 2, 3, 6-tri-O-methyl derivatives[3] significantly improves aqueous solubility and enhances complexation ability.[4] However, the preparation of defined methyl derivatives of β-CD is not without chemical problems related to the difference in the reactivity of the hydroxyl groups at positions 2, 3 and 6 of cyclodextrin rings.[5] It is therefore necessary to be able to follow the progress of the reaction, to calculate the reaction yield and to characterise the obtained products.

Various high performance liquid chromatographic (HPLC) methods have been applied for maltodextrin and cyclodextrin analysis using such columns as aminopropyl-bonded,[6-11] aminocyano-bonded,[12] $C_8$ and $C_{18}$-bonded ,[7,11,13-17] and ion exchange columns.[18-21] More recently the use of graphitized carbon columns has been reported.[22]

The main difficulties during the analysis of carbohydrates are caused by the limitations of the detection systems, due to the absence of UV chromophore groups in cyclodextrins and maltodextrins. Several means of detection have been employed including refractive index,[6,7,9,11-13,16,19,22] pulsed amperometric,[21,23] fluorescence,[24] polarimetric [9,11] and indirect photometric detection.[25]

Both refractive index detection and polarimetric detection are very sensitive to changes in the refractive index of the mobile phase caused by variations in temperature, pressure and mobile phase composition.[26] As a result, the lack of sensitivity of these detection modes and the impossibility of carrying out gradient elutions with these detectors on one hand and the complexity of the derivatisation systems on the other, have given rise to the development of light scattering detection.[10,20,27]

In fact, the evaporative light scattering detector (ELSD) which, like refractometry, is a universal detector, is even more sensitive,[28] easy to use (because of the absence of base-line drift and its rapid equilibration), and compatible with gradient elution. It has also been shown to be a good choice for analytical studies of carbohydrates [29] and more recently for cyclodextrin analysis.[27]

In this paper, ELSD performances for cyclodextrin analysis are described. The limits of detection, response factors and quantitative impurity results obtained are reported. Different isocratic and gradient LC systems have been optimized to control the purity of commercial β-CD and TM-β-CD.

## EXPERIMENTAL

### Apparatus

The liquid chromatographic apparatus consisted of a Varian (Palo Alto, CA, USA) Model 9010 gradient pump, a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a 20-µl sample loop and two types of detectors: an Evaporative Light Scattering Detector (ELSD) (Sedere, Alfortville, France) Model Sedex 55 and a Refractive Index Detector (Showa Denko, Tokyo, Japan) Model Shodex RI-71.

The usual ELSD detector settings were as follows: photomultiplier, 9; evaporative temperature, 30°C; air pressure, 2.2 bar. Data were processed using a Shimadzu (Kyoto, Japan) Model CR 5A integrator.

The following columns were used: Zorbax Phenyl (250 x 4.6 mm I.D.) (Rockland Technologies, INC., Newport, DE, USA) and Nucleosil Phenyl (150 x 4.6 mm I.D.) (Macherey-Nagel, Düren, Germany). The flow rate was 1 mL/min, experiments were carried out at room temperature.

### Reagents

Acetonitrile (RS for LC) was purchased from Carlo Erba (Milan, Italy) and water from the Elgastat UHQ II System from Elga (Antony, France).

Glucose and maltose were obtained from Merck (Darmstadt, Germany), heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD), maltotetraose, maltopentaose, maltohexaose and maltoheptaose from Sigma (S' Louis, MO, USA), α-, β-, γ-CD from Wacker (Lyon, France), heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) from our laboratory.

The sample solvent for maltodextrins and α-, β-, γ-CD was pure water and 50:50 acetonitrile-water for DM-β-CD and TM-β-CD.

## RESULTS AND DISCUSSION

### ELSD Performances for Cyclodextrin Analysis

ELSD performances for cyclodextrin analysis have been studied in order to optimize the cyclodextrin response, to compare the responses of cyclodextrins and to obtain a crude estimation of the linearity of the detection system and an approximation of the detection threshold.

The principle of the Evaporative Light Scattering Detector (ELSD) is to nebulize the column effluent into droplets which are carried by a nebulizing gas (air, nitrogen, etc.) into an evaporator tube and then directed towards a light beam. Light is scattered by residual particles of non-volatile analyte and measured by a photomultiplier, providing the chromatographic signal.[30]

Column effluent nebulization favours the elimination of the solvent constituting the mobile phase while avoiding partial vaporization of the solute. Increasing the evaporator tube temperature causes solutes which possess a moderate or high molar volatility to evaporate and, consequently, the scattered light has a lower intensity.

### Detector Response

Table 1 reports peak-areas of cyclodextrins and glucose according to the temperature of the detector evaporator tube.

**Table 1**

**Peak-Area of α-, β-, γ-CD, TM-β-CD and Glucose According to the Temperature of the Detector Evaporator Tube**

| | | Evaporator Tube Temperature | | | $\frac{\Delta A^*}{A_{30}}$ |
|---|---|---|---|---|---|
| | | 30°C | 50°C | 80°C | |
| Glucose | $tr_{min}$ | 1.88 | | | |
| | Peak-area | 6 100 256 | 5 358 328 | 4 715 938 | 23 % |
| α-CD | $tr_{min}$ | 2.21 | | | |
| | Peak-area | 6 784 958 | 6 639 871 | 6 038 746 | 11% |
| γ-CD | $tr_{min}$ | 4.44 | | | |
| | Peak-area | 5 424 940 | 5 188 351 | 4 869 416 | 10 % |
| β-CD | $tr_{min}$ | 6.20 | | | |
| | Peak-area | 4 166 424 | 4 148 751 | 3 955 971 | 5 % |
| TM-β-CD | $tr_{min}$ | 8.60 | | | |
| | Peak-area | 8 207 192 | 6 576 868 | 6 083 077 | 26 % |

* $\frac{\Delta A}{A_{30}} = \frac{A_{30} - A_{80}}{A_{30}}$ x 100; $A_{30}$, $A_{80}$ are the peak-area values for each compound at 30°C and 80°C.

Column, Nucleosil Phenyl (150 x 4.6 mm I.D.); mobile phase, 100 % water for glucose, acetonitrile-water (5:95) for α-, β-, γ-CD, acetonitrile-water (40:60) for TM-β-CD; flow-rate, 1 mL/min; concentration of each solute, 500 mg/L.

The composition of the injection solvent and of the mobile phase was the same; ELSD Sedex 55.PM = 9, P = 2.2 bar.

Whatever the retention of solutes (near the void volume or with a great retention), we noted that the response of cyclodextrins, like that of glucose, decreased with increasing temperature from 30°C to 80°C. The best detector response is with a vaporization tube temperature of 30°C.

TM-β-CD, like glucose, is subject to a higher variation of peak-area according to the temperature, than the native cyclodextrins (α-, β- and γ-CD). Values of $\frac{\Delta A}{A_{30}}$ clearly depict this result.

For all studied solutes, the best detector response is obtained with a vaporization tube temperature equal to 30°C. This result is in good agreement with that already published for sugars, i.e. a low evaporator tube temperature is better than a high temperature.[31]

For given detector parameters, TM-β-CD like α-, β- and γ-CD, has a better response than glucose. For a given mobile phase composition (acetonitrile-water, 5:95), for α-, β- and γ-CD, the more the compound is retained on the stationary phase, the lower the peak-area is. However, when these same solutes are eluted near the void volume by increasing the acetonitrile percentage in mobile phase, their detector response is nearly equal. So, these differences seem to depend on the detection system rather than on the nature of the solute. The evaporation yield decreases when the solute retention increases. These results are in good agreement with those commonly obtained with series of homologous compounds.[32,33]

For a sample concentration of 500 mg/L, TM-β-CD has a higher detector response than β-CD although its retention time was greater than that of β-CD (Table 1). Two reasons can explain this fact: firstly, elution of TM-β-CD requires a greater amount of acetonitrile (40 %) in the eluent than elution of β-CD (5 %). Therefore, the mobile phase is easier to evaporate in the case of TM-β-CD elution. Consequently, nebulization yield is better (the more the organic solvent percentage increases in the mobile phase, the more the nebulization yield increases) and the size of droplets may be lower.[30] So, detector sensitivity is better for TM-β-CD than for α-, β-, γ-CD. Secondly, the methylation of all hydroxyl groups of β-CD to obtain TM-β-CD has modified the physical properties of β-CD. This is confirmed by the fact that, when β-CD and TM-β-CD are eluted near the void volume with the same mobile phase, the TM-β-CD response is higher than the β-CD one.

**Calibration Curves**

In order to achieve accurate quantitative analysis of α-, β-, γ-CD and TM-β-CD, calibration curves have been carried out with water-acetonitrile eluent on a Nucleosil Phenyl column in an isocratic mode. Although the variation of

**Table 2**

**Calibration Curves Log A* = b Log C* + Log a for α-, β-, γ-CD, and TM-β-CD Studied Concentration Range: 2.5 to 1000 mg/L**

| Solute | b* | Log a* | Correlation Coefficient | Retention Time (min) |
|---|---|---|---|---|
| α-CD | 1.38 | 6.98 | 0.9994 | 2.2 |
| γ-CD | 1.40 | 6.70 | 0.9994 | 4.3 |
| β-CD | 1.42 | 6.33 | 0.9994 | 6 |
| Average value | 1.40 | 6.67 | 0.9994 | --- |
| R.S.D. (%) | 1.43 | 4.89 | --- | --- |
| TM-β-CD | 1.67 | 5.35 | 0.9993 | 8.7 |

*Where A is the measured peak-area, C is the cyclodextrin concentration, b is the slope, and Log a is the γ-intercept.
Column, mobile phase and detection as in Table 1.

the ELSD response is complex (it depends on droplet size, concentration and nature of solute, gas and liquid flow rates, vaporization temperature, etc.), it was assumed that in a large range of sample size the measured peak area can be related to sample size by the following relationship:[34]

$$A = a C^b$$

Where b is the slope of the response line, C is the solute concentration and $a$ is the response factor. As a result, the linearity between surface area response and concentration is obtained in double logarithmic coordinates.[35] (Curve parameters are reported in Table 2.).

$$\text{Log}\, A = b \,\text{Log}\, C + \text{Log}\, a$$

For the four cyclodextrins studied, graphs were linear with an acceptable correlation coefficient ($R \approx 0.9994$). This result was in agreement with the quantitative determination of sugars.[29] The linear dynamic range is almost 3 decades (concentrations vary from 2.5 to 1000 mg/L).

Slope b mentioned in the literature has values generally comprised between 1 and 1.6, with 1.3 being the most representative value.[29] Slopes obtained for α-, β- and γ-CD are contained in the expected interval and are close to the most representative value 1.3 and also to those of sugars, for which the slope is 1.24 [29] (average slope for α-, β- and γ-CD is 1.40).

It has been observed by different authors that within a group of homologous compounds, the detector response is nearly equal.[34,35] Considering the low value of the relative standard deviation of slope b (R.S.D. = 1.43 %) and of the intercept Log a (R.S.D. = 4.89 %), the detector response can be considered as equal for α-, β- and γ-CD. On the other hand, for TM-β-CD, the slope is slightly higher ($b$ = 1.67) and the intercept is slightly lower (Log a = 5.35) than those of α-, β- and γ-CD. These variations of curve parameters seem due, like variations on peak-area, to the eluent composition (richer in acetonitrile for TM-β-CD elution than for α-, β- and γ-CDs elution) and to modifications of TM-β-CD physical properties.

We concluded that, in order to achieve a more accurate determination of impurities, it was necessary to make one calibration curve specially for TM-β-CD.

**Repeatability**

The repeatability of the analysis which is expressed by the relative standard deviation of the area of each cyclodextrin peak has been also evaluated (Table 3). The repeatability determined from five replicate injections was satisfactory for all the cyclodextrins studied (α-, β-, γ-CD and TM-β-CD): the deviations varies only from 1.14 % to 3.58 %.

**Detection Limits**

Table 4 lists detection limits for α-, β-, γ-CD and TM-β-CD obtained with the RI and with the ELSD. Using the most sensitive range of the ELSD, a 50 ng (20 µl of 2.5 mg/L) detection limit of α-cyclodextrin and 80 ng (20 µL of

**Table 3**

**Repeatability for Standard Solutions of α-, β-, γ-CD, and TM-β-CD**

| Solute | α-CD (350mg/L) | β-CD (350 mg/L) | γ-CD (500 mg/L) | TM-β-CD (300 mg/L) |
|---|---|---|---|---|
| Peak-area | 4 166 611 | 4 339 606 | 5 452 070 | 3 881 604 |
| | 4 155 605 | 4 381 030 | 5 366 712 | 4 057 117 |
| | 4 019 240 | 4 271 929 | 5 305 771 | 3 970 371 |
| | 3 957 053 | 4 248 159 | 5 330 156 | 3 987 729 |
| | 4 218 230 | 4 198 303 | 5 423 002 | 3 693 827 |
| Mean | 4 103 348 | 4 287 805 | 5 375 542 | 3 918 130 |
| R. S. D. (%) | 2.68 | 1.70 | 1.14 | 3.58 |

Column, mobile phase and detection as in Table 1.

**Table 4**

**Comparative Detection Limits for α-, β-, γ-CD, and TM-β-CD with a RI and an ELS Detector**

| | ELS Detector | RI Detector |
|---|---|---|
| **Solute** | **Detectable mass on column*(ng)** | |
| α-CD | 50 | 2 000 |
| β-CD | 80 | 2 000 |
| γ-CD | 80 | 2 000 |
| TM-β-CD | 120 | 1 000 |

*When signal-to-noise ratio (S/N) equals 3.
Column, Nucleosil Phenyl (150 x 4.6 mm I.D.); mobile phase, acetonitrile-water (5:95) for α-, β- and γ-CD, acetonitrile-water (40:60) for TM-β-CD; compounds are in solution in the eluent.

4 mg/L) detection limit of β- and γ-cyclodextrins are obtainable, at a signal-to-noise ratio of 3. So, it is seen that the minimal detectable quantity achieved by the ELSD is much lower than that of the RI detector (2 000 ng for α, β, γ-CD and 1 000 ng for TM-β-CD with the RI detector). The detection thresholds we have obtained with the Shodex RI-71 detector were comparable to those obtained with another RI detector previously published by G. White et al.[12] The minimal detectable quantity achieved with the polarimetric detector is also higher than that of the ELSD.

Indeed, the detection limit obtained for TM-β-CD with the polarimetric detector was 2000 ng.[11] So, the level of sensitivity of the ELSD was suitable to provide a satisfactory impurity profile of cyclodextrins.

We can conclude that the ELSD is suitable for performing the assay of impurities in cyclodextrins to ensure quality in these products.

**Analysis of a Commercial β-CD**

The β-CD is selectively purified from the crude digest of starch containing linear glucose polymers (glucose to maltoheptaose), α-CD and γ-CD. So, an analytical method allowing the separation of these compounds, characterized by very different polarities, was necessary. Moreover, to obtain the best of all possible sensitivities, the analytical method has to permit elution of all potential impurities before the major product.

The separation of linear glucose polymers and cyclodextrins has already been studied on amino [7,8] and amino-cyano [12] columns with acetonitrile-water as mobile phase. Detection is commonly performed by RID, so analyses must be performed isocratically. In these chromatographic conditions, cyclodextrins are eluted with elution times comparable to those of maltooligosaccharides. Besides, the overlapping of cyclodextrin and maltodextrin chromatographic peaks prevents the determination of small amounts of impurities in the presence of a large proportion of β-CD and leads to a reduction in sensitivity.

On the other hand, cyclodextrins are strongly adsorbed on octadecyl bonded silica column.[29] So, on this type of support, the percentage of organic modifier in the aqueous-acetonitrile mobile phase necessary to elute these compounds was so high that linear sugars were eluted near the void volume in these conditions.

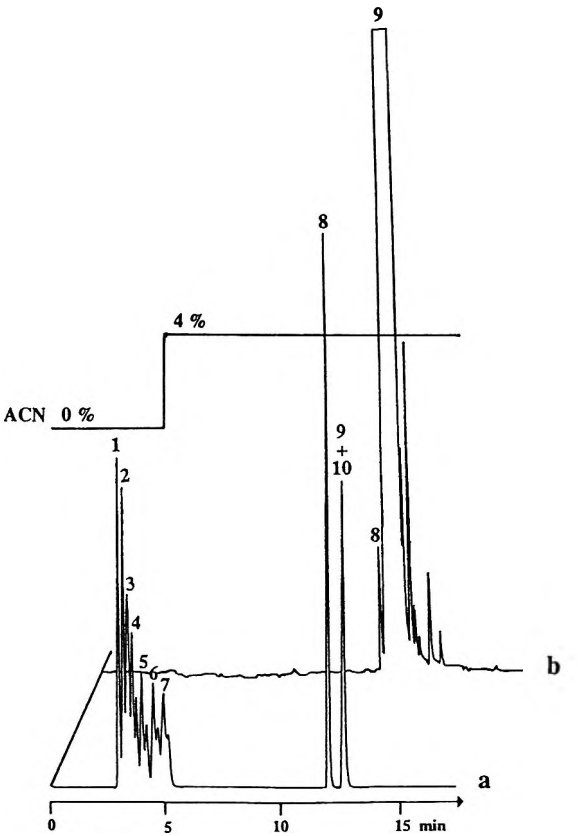


**Figure 1**. (a) Analysis of a standard mixture of β-cyclodextrin and nine potential impurities. (b) Analysis of a commercial β-cyclodextrin (10 000 mg/L). Column: Zorbax Phenyl (250 x 4.6 mm I.D.); gradient elution, water during 5 min, then acetonitrile-water (4:96) during 10 min ; flow-rate, 1 mL/min ; evaporative light scattering detector.
Elution order: 1, glucose; 2, maltose; 3, maltotriose; 4, maltotetraose; 5, maltopentaose; 6, maltohexaose; 7, maltoheptaose; 8, α-CD; 9, β-CD; 10, γ-CD.

With a lower acetonitrile percentage, good separation of malto-oligosaccharides was observed but cyclodextrins are not eluted. Simultaneous analysis of cyclodextrins and maltodextrins on octadecyl bonded silica column requires gradient elution and the use of a detector compatible with this elution mode.

Our previous study, concerning the RP-LC analysis of methylated β-CD[27] has showed that phenyl bonded silica column offers selectivities that are different from those obtained using classical $C_{18}$-bonded silica column and allows to achieve satisfactory isocratic analysis of these compounds with a shorter time.

In this paper, Fig. 1a shows the separation of a standard mixture of β-CD and of its potential impurities which included 7 linear (glucose to maltoheptaose) and 2 unbranched cyclic (α- and γ-CD) glucose polymers. The compounds are detected by ELSD, following separation on a Zorbax phenyl column with an acetonitrile-water gradient. This gradient consists in 100 % of water during 5 min, then step gradient to acetonitrile-water (4:96) during 10 min.

An entirely aqueous medium was necessary to retain and to separate glucose to maltoheptaose. Elution occurs in order of increasing molecular mass. Despite their short elution times (tr ≤ 5 min), the α- and β-anomers of maltotetraose, maltopentaose, maltohexaose and maltoheptaose are resolved. On the other hand, a small amount of acetonitrile was necessary to elute cyclodextrins which show more hydrophobic interaction than linear sugars. So it is easier, than on amino columns, to separate the cyclic glucooligomers from the linear glucooligomers.[12] Consequently, phenyl bonded-silica column with gradient elution provided an efficient means of isolating cyclodextrins from maltooligosaccharides because of the great difference of retention of these compounds. In addition, the use of gradient elution allows a rapid separation of the compounds (<13 min).

To show the potential of this new method, a commercial sample of β-CD (10 000 mg/L in 100 % water) was analyzed in these chromatographic conditions (Fig. 1b). The commercial β-CD did not exhibit any of the linear glucose impurities. At the end of the analysis, by increasing the percentage of acetonitrile to 20 %, we ascertained the absence of other non-eluted impurities in the chromatographic conditions of the standard mixture analysis. However, the batch contained a small amount of α-CD and of other impurities which are eluted after β-CD.

This system did not permit the γ-CD chromatographic peak to show because β- and γ-CD are not resolved in these conditions. So another chromatographic system has been studied in order to improve separation between β- and γ-CD.

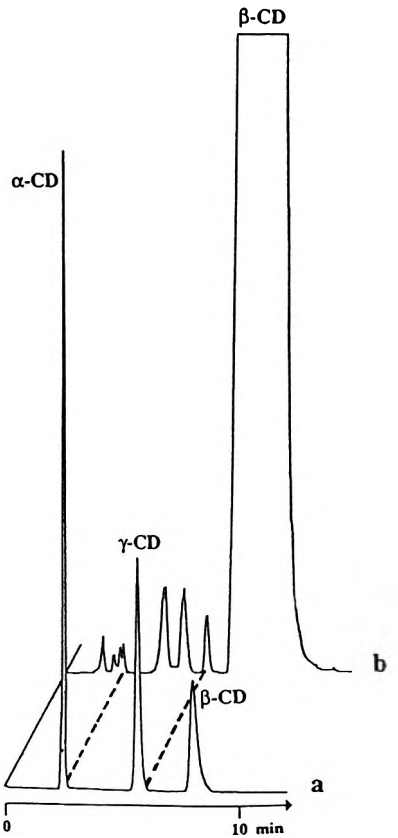


**Figure 2**. (a) Analysis of a standard mixture of α-, β-, and γ-cyclodextrin. (b) Analysis of a commercial β-cyclodextrin (10 000 mg/l). Column, Nucleosil Phenyl (150 x 4.6 mm I.D.); eluent, acetonitrile-water (4:96) (v/v); flow-rate, 1 mL/min; evaporative light scattering detector.

Fig. 2a depicts a chromatogram illustrating the high resolution ($R_s \geq 4$) and rapid separation (< 9 min) of α-, β- and γ-CD on a Nucleosil phenyl column in isocratic mode with acetonitrile-water (4:96) as eluent. On a Nucleosil phenyl column, the elution order is the same as on $C_{18}$-bonded silica columns:[11,17] β-CD is more retained than γ-CD which is more retained than α-CD. But the advantage of this system with regard to $C_{18}$ columns is that the chromatographic analysis is shorter and efficiencies are better.

On Phenyl bonded silica column (Nucleosil or Zorbax), acetonitrile-water mixtures were found to be very suitable solvents for developing the chromatograms of cyclodextrins, whereas on $C_8$ or $C_{18}$-columns, aqueous methanolic or ethanolic solutions provide better results than acetonitrile-water mixtures.[11]

The commercial β-CD (10 000 mg/L) has also been analyzed in these chromatographic conditions. Fig. 2b shows that the sample of β-CD did indeed contain a small amount of α-CD but also a small amount of γ-CD. We could also detect two other unidentified impurities which are eluted between α- and γ-CD (in the previous system, they were eluted with the peak of β-CD). These results prove that the two systems are complementary: with Zorbax Phenyl, characterization of linear sugars traces and of impurities which are more hydrophobic than β-CD is possible, whereas linear sugars are eluted near the void volume without separation on Nucleosil Phenyl even if 100 % water is used as mobile phase. With Nucleosil Phenyl, the high selectivity between α- and β-CD allows detection of γ-CD traces which are eluted with the same retention as β-CD on Zorbax Phenyl.

Calibration curves previously established have allowed the quantification of the proportion of α- and γ-CD in β-CD. The commercial β-CD contains about 0.05 % (w/w) of α-CD and about 0.2 % (w/w) of γ-CD. Considering all of the impurities and the fact that they have an ELSD response close to that of β-CD, the degree of β-CD purity could be estimated at 99.3 %.

So Nucleosil phenyl column with acetonitrile-water mobile phase is a suitable system to quantify small amounts of α- and γ-CD in the presence of a large proportion of β-CD.

**Analysis of a Commercial TM-β-CD**

A similar study has been carried out to determine the potential impurities of TM-β-CD in which the three OH groups of every glucose unit are replaced by an $O\text{-}CH_3$ group. In view of the complexity of β-CD alkylation methods, an analytical method was needed in order to determine the degree of purity of commercial and synthetic samples.

Investigations into the analysis of methylated and partially methylated cyclodextrins by HPLC have already been reported.[24,36-39] The methyl derivatives of β-CD are much more strongly adsorbed on hydrophobic

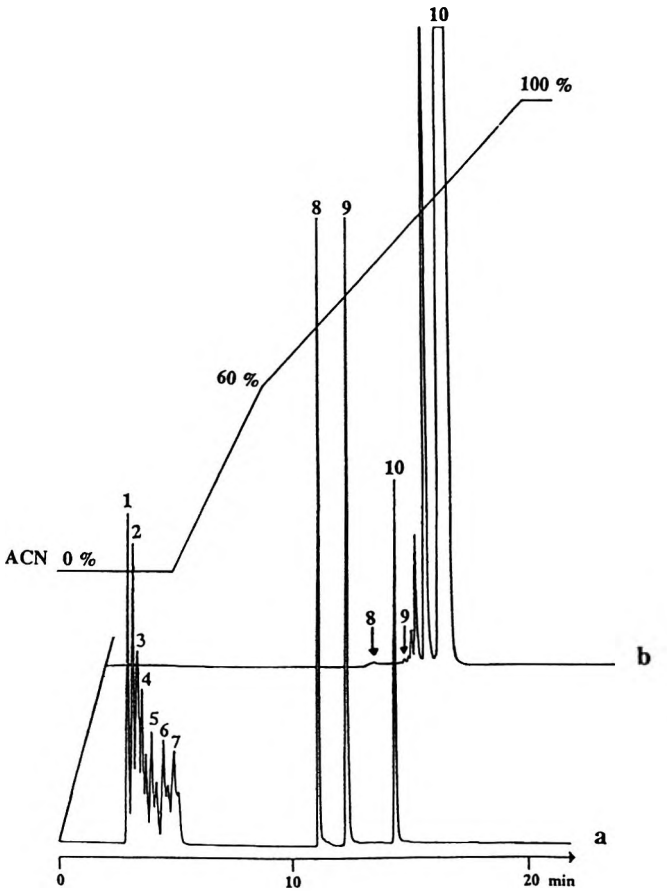


**Figure 3**. (a) Analysis of a standard mixture of TM-β-CD and nine potential impurities. (b) Analysis of a commercial TM-β-CD (10 000 mg/l). Column, Zorbax Phenyl (250 x 4.6 mm I.D.); eluent, acetonitrile-water : water during 5 min, followed by gradient elution from 0 % to 60 % acetonitrile in 4 min, then to 100 % acetonitrile in 11 min; flow-rate, 1 mL/min; evaporative light scattering detector.
Elution order: 1, glucose; 2, maltose; 3, maltotriose; 4, maltotetraos; 5, maltopentaose; 6, maltohexaose; 7, maltoheptaose; 8, β-CD; 9, DM-β-CD; 10, TM-β-CD.

stationary phases than the parent β-CD itself. Their elution requires higher concentrations of organic additive in the aqueous mobile phase solutions. So, simultaneous analysis of compounds with such widely differing polarities as linear sugars, β-CD and methylated β-cyclodextrins requires gradient elution which precluded the use of an RI detector.

Figure 3a presents the chromatogram of a standard mixture of β-CD, DM-β-CD, TM-β-CD and also linear sugars (glucose to maltoheptaose). These compounds are detected by ELSD after being separated on a Zorbax Phenyl column with an acetonitrile-water gradient. Mobile phase consists in water during 5 min, followed by gradient elution from 0 % to 60% acetonitrile in 4 min, then to 100 % acetonitrile in 11 min. Gradient elution with a rapidly increasing acetonitrile content, compatible with the ELSD, enables excellent separation. As on a $C_{18}$-bonded silica, the more methylated the compound is, the more its retention time increases. Contrary to the separation of β-CD, DM-β-CD and TM-β-CD obtained on $C_{18}$-bonded silica column with a polarimetric detector,[11] separation on Zorbax Phenyl column with the ELSD is obtained without baseline drift, with good efficiencies and in a short time (tr < 15 min). The separation enables a rapid qualitative determination of the TM-β-CD impurities.

Figure 3b shows the chromatographic fingerprint of a commercial TM-β-CD eluted in the same conditions as the standard mixture. The commercial TM-β-CD did not show any of the linear glucose impurities and contained very slight amounts of β-CD and DM-β-CD. Other chromatographic peaks, however, are present between the DM-β-CD peak and TM-β-CD peak. In view of the complexity of the β-CD alkylation method, these compounds should correspond to over-methylated homologues of DM-β-CD. To increase the resolution between DM-β-CD and TM-β-CD and thus to obtain a better distribution of partially methylated cyclodextrin chromatographic peaks, another chromatographic system has been studied.

Fig. 4a shows a very good isocratic separation of the standard mixture of β-CD, DM-β-CD and TM-β-CD on Zorbax Phenyl column with acetonitrile-water (45:55) as mobile phase. This isocratic analysis is very interesting because, until now, such a mixture could be analyzed only with a gradient elution on $C_{18}$-bonded silica column.[11] Under such elution conditions, β-CD was eluted close to the void volume and selectivity between DM-β-CD and TM-β-CD has significantly been improved in comparison with the gradient analysis. This system allows us to confirm that there is no β-CD and DM-β-CD in the commercial TM-β-CD but there are a high amount of compounds which have an intermediate methylation degree between those of DM-β-CD and TM-β-CD (Fig. 4b). By using the TM-β-CD calibration curve, the evaluation of the major impurity proportion was about 4.5 % (w/w). Considering all of the impurities, the degree of purity of TM-β-CD was about 94 %. So, commercially, it is more difficult to obtain a pure methylated cyclodextrin than its corresponding native cyclodextrin.

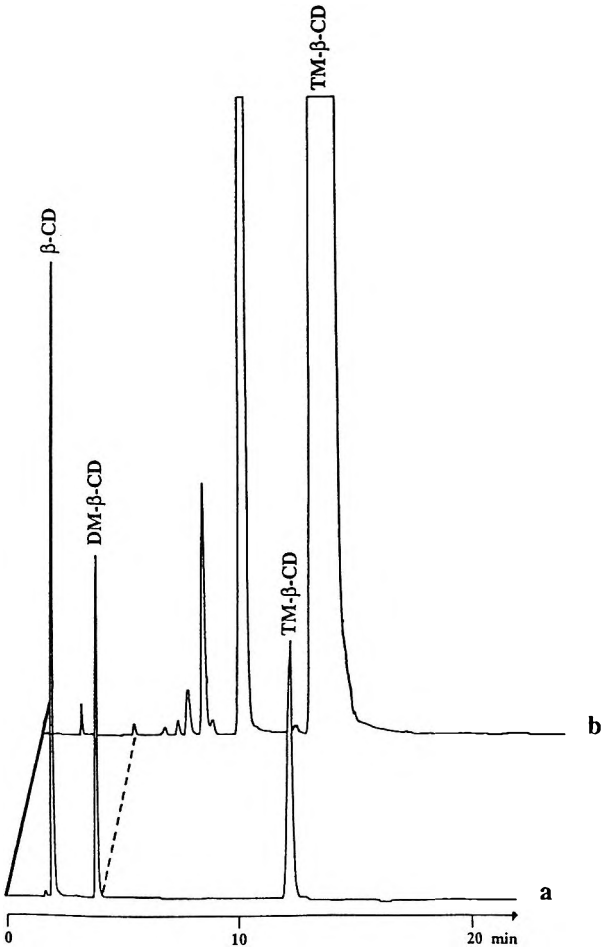


**Figure 4**. (a) Analysis of a standard mixture of β-CD, DM-β-CD and TM-β-CD. (b) Analysis of a commercial TM-β-CD (10 000 mg/L). Column, Zorbax Phenyl (250 x 4.6 mm I.D.); eluent, acetonitrile-water (45:55); flow-rate, 1 mL/min; evaporative light scattering detector.

## CONCLUSION

This work presents the first application of the evaporative light scattering detector used to determine the purity of native (α-, β-, γ-CD) and derivatised (TM-β-CD) cyclodextrins. Although, strictly speaking, the response of ELSD is not directly proportional to the mass of the solute, the ELSD response factors

of these homologous compounds are highly similar, so it is possible to evaluate impurity levels, with an acceptable degree of approximation, without previous identification.

The use of ELSD permits one to obtain the separation of compounds with widely differing polarities, such as linear sugars, β-CD, and methylated β-CDs, as a result of its compatibility with gradient elution mode. Separation of cyclodextrins using Phenyl-bonded stationary phase and acetonitrile-water mobile phase offers selectivities that are different from those obtained using classical $C_{18}$-bonded silica column and allows one to achieve satisfactory isocratic and gradient analysis. The method provides good selectivity and sensitivity for the determination of cyclodextrins. Moreover, chromatographic systems presented here allowing the elution of impurities before the major product, could be used on the semi-preparative scale to purify TM-β-CD and can be used to compare different sources of β-CD and TM-β-CD and to measure batch to batch variation.

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# ANALYSIS OF QUATERNARY AMMONIUM COMPOUNDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH EVAPORATIVE LIGHT SCATTERING DETECTION

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## ABSTRACT

A high performance liquid chromatographic (HPLC) procedure employing an Evaporative Light Scattering Detector (ELSD) for the analysis of quaternary ammonium compounds in ethnic hair care formulations is reported.

## INTRODUCTION

Quaternary ammonium compounds (quats) comprise a great number of materials used primarily as surfactants and antimicrobials. They are used in applications ranging from laundry softeners to phase-transfer catalysts and hair conditioners. Chemically, these compounds are substituted ammonium salts, where the nitrogen atom is covalently bound to four alkyl or aryl groups. The result is a nitrogen atom with a net positive charge that maintains its character regardless of the surrounding pH.

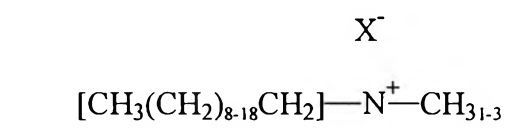



**Figure 1.** Simplified Structure of Typical, Commercially Available Quaternary Ammonium Mixture

The associated anionic component of a quat can be a bromide, chloride or methosulfate ion. Quats are usually marketed with a solvent which can be water, isopropyl alcohol, propylene glycol or sold as a flake to make handling easier.

Analysis of quaternary ammonium compounds is difficult by reverse phase HPLC due to the nature of their composition. The alkyl portion of these compounds is typically a homologous series of C8 through C20 varying in percent composition based upon the source used in production. This means that commercial quaternary ammonium compounds are comprised of many similar molecules with a distribution based upon the aliphatic chain length (see Figure 1). This type of molecular distribution actually enhaces the effectiveness of surfactants. Being a surfactant, the quaternary ammonium molecule has both hydrophobic and hydrophilic moieties. Furthermore, direct determination of these quaternary surfactants in their various sample matrices is complicated.

Many HPLC methods for the determination of quaternary alkylammonium compounds have been reported. These methods generally fall into the categories of environmental recovery and quality control. Wee and Kennedy reported a normal phase method for the determination of cationic surfactants without separation of the homologous series in environmental samples.[1] This method has been continuously improved by others.[2-4] Many HPLC methods exist for the determination of alkylammonium compounds in which the homologous series is resolved.[5-7]

Additionally, other HPLC methods have been reported for the determination of alkylammonium compounds in pharmaceuticals in which the homologous series may or may not be resolved.[8-11] A suitable method for the quality control of quaternary ammonium compounds in cosmetic formulations was reported by Matsuzaki and others.[12]

The requirement of the reported analysis was that the homologous series of the quaternary ammonium compounds not be resolved. The goal was to elute the series as a single peak separated from all other components in the sample matrix. The charge of the quaternary amine appeared the most logical approach to obtain the goal. It followed that the selectivity of ion-exchange chromatography would elute the homologous series as a single peak.

The selectivity of organic compounds in cation-exchange chromatography is complex. Extraneous to true ion exchange, hydrophobic interactions between the polymer backbone of the ion exchanger and the fatty portion of the ion, as well as solvation, play a major role in selectivity of organic ions.

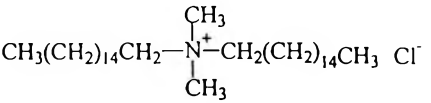
Dumont, Fritz and Schmidt reported the use of non-aqueous solvents in cation-exchange for the selective separation of protonated primary, secondary and tertiary amines.[13] By using strictly non-aqueous mobile phases, the hydrophobic interactions and solvation effects were minimized. These workers concluded that the size and shape of the cation may affect ion-exchange selectivity. Alternatively, Spagnolo and others, reported an ion-exchange HPLC method using non-aqueous buffers.[14]

Similarly, the described methods have been modified to demonstrate selectivity for several different types of quaternary alkylammonium chlorides in a typical hair relaxer formulation. The new method disclosed here is a selective, precise and accurate method for the determination of alkylammonium chlorides in a typical hair relaxer formulation.

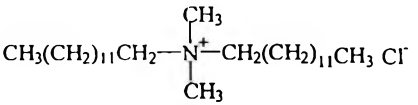
## EXPERIMENTAL

### Chemicals and Reagents

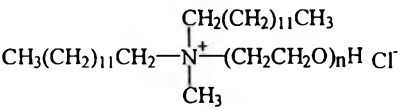
HPLC grade acetonitrile, HPLC grade methanol, and reagent grade ammonium formate all were purchased from Fisher Scientific. N-Hexadecyl-N,N-dimethyl-1-hexadecanaminium chloride (Varisoft 432 PPG, 68.02%), N-Tridecyl-N,N-dimethyl-1-tridecanaminium chloride (Varisoft 2TD, 74.5%), Polyethylene glycol (5) ditridecylmonium chloride (Varisoft 5TD, 56.62%), and N,N-Dimethyl-N-octadecylbenzenemethanaminium chloride (Varisoft SDC-85, 86.7%) were obtained from Witco Corp. The structures of the alkylammonium chlorides are given in Figure 2.



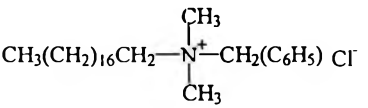
**Varisoft 432 PPG**



**Varisoft 2TD**



**Varisoft 5TD**



**Varisoft SDC-85**

**Figure 2**. Aklyammonium Chlorides Under Investigation.

In this study, a typical base phase of a two-part hair relaxer formulation was examined. The materials used in this formulation were as follows: Deionized water, polyoxypropylene (12) polyoxyethylene (65) lanolin oil, petroleum jelly, liquid paraffin, polyoxyethylene (20) cetyl/stearyl ether, 1-hexadecanol, 1-octadecanol, and calcium hydroxide. To this blend was added 3% actives of each experimental quat (see Figure 2.). Hair relaxers are formulated to a pH range of 11-13 to effectively modify the secondary protein structure of hair.

## Apparatus

The chromatographic system consisted of a Thermo Separation Products (TSP) P4000 pump, TSP AS3000 autosampler, TSP 4400 interface, TSP inline

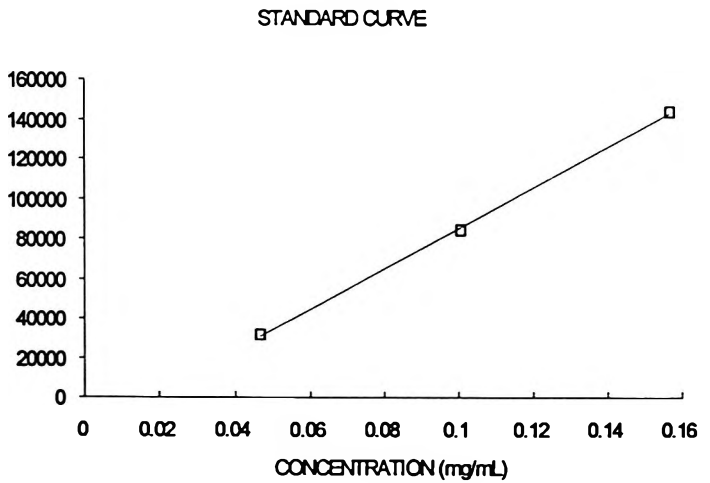



**Figure 3**. Typical Standard Curve with Linear Regression Shown.

membrane degasser, Varex MKIIIA ELSD set at 85°C and 2.4 SLPM (standard liters per minute), and TSP PC1000 data acquisition system. A Metachem Spherisorb SCX (150 x 4.6 mm, 5 μM) was maintained at ambient temperature.

## Mobile Phase

The mobile phase consisted of a methanol buffer containing 0.06M ammonium formate. The mobile phase was filtered through a .45 μm filter (Gelman Sciences, Supor-460, 47 mm) and degassed. The flow rate was 1.0 mL/min.

## Preparation of Standards

Stock solutions were prepared by accurately weighing Varisoft 432 PPG, Varisoft 2TD, Varisoft 5TD and Varisoft SDC-85 standards into separate 50 mL volumetric flasks and diluting to volume with acetonitrile.

Standard solutions were prepared by pipeting 1, 2 and 3 mL of the stock solutions into separate 10 mL volumetric flasks and diluting to volume with acetonitrile. A standard curve was generated in the range from 200 to 600 ppm (Figure 3).

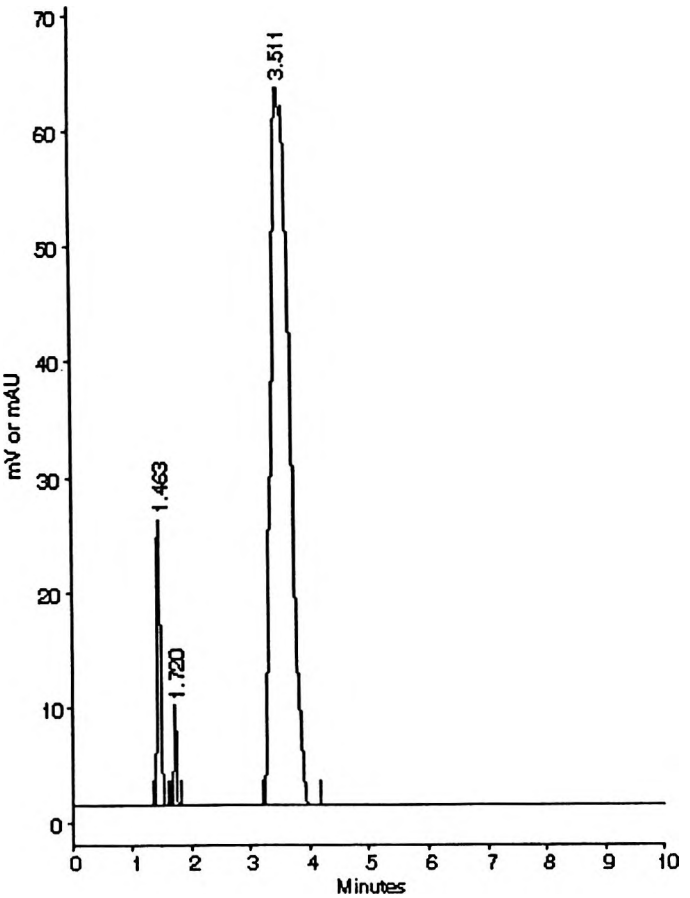



**Figure 4**. Varisoft 432 PPG Standard.

**Preparation of Samples**

The sample was placed in a 55°C oven for 15 minutes. The sample was removed, shaken vigorously and *ca.* 100 mg tranferred into a 10 mL volumetric flask in triplicate. These samples were diluted to volume with acetonitrile, sonicated (10 min), shaken (manually) and sonicated (10 min). The samples were left overnight to prevent leaching difficulties. The following day the samples were sonicated (10 min), shaken (manually) and filtered through a .45μM PTFE syringe filter (Alltech Cat. #2386). This sample preparation selectively "floated" the sample matrix in acetonitrile, thereby, extracting the quat.

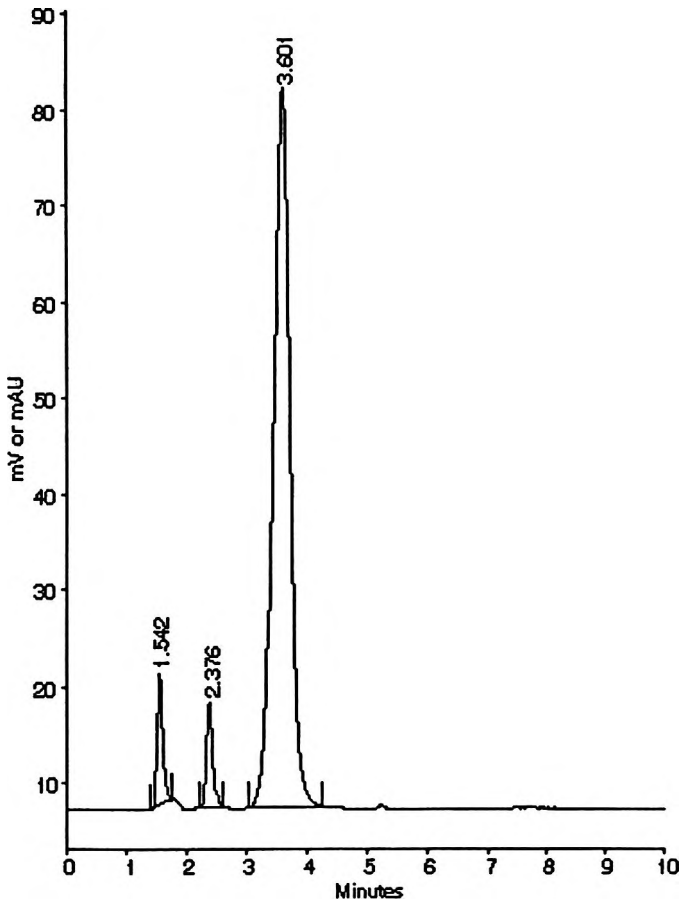



**Figure 5**. Varisoft 2TD Standard.

## RESULTS AND DISCUSSION

### Chromatography

Chromatograms resulting from a 20 mL injection of each standard preparation are given in Figures 4-7. The data acquisition time was 10 minutes for all injections.

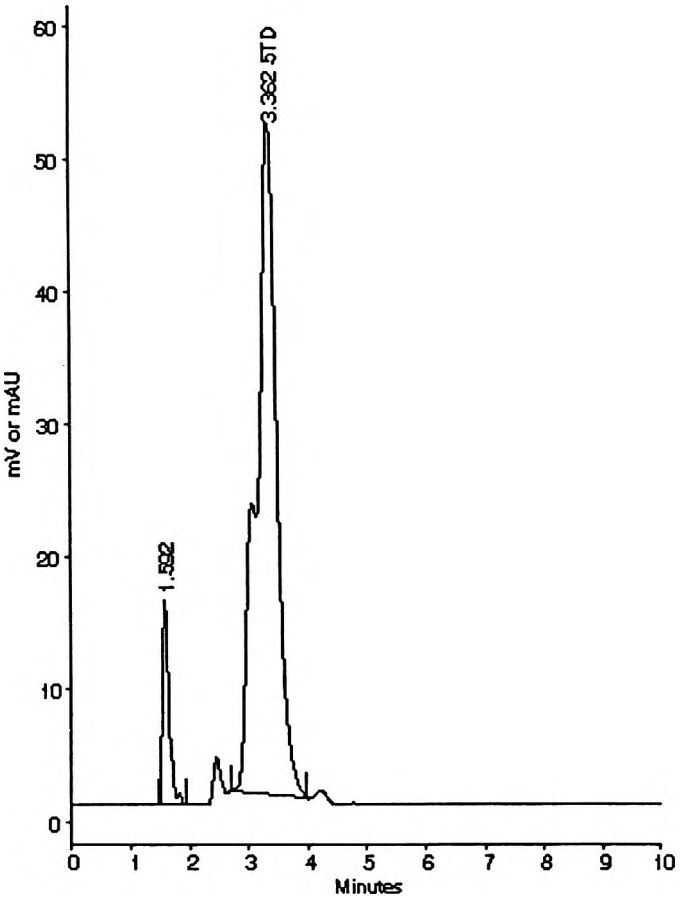



**Figure 6**. Varisoft 5TD Standard.

## System Suitability

Because an ELSD gives a linear response over a narrow range, a three point calibration curve was prepared for each quat each day. A correlation coefficient of 0.999 was the minimum requirement. To ascertain system precision, seven replicate injections of a sample preparation containing Varisoft SDC-85 were assayed and gave an RSD of 0.4%.

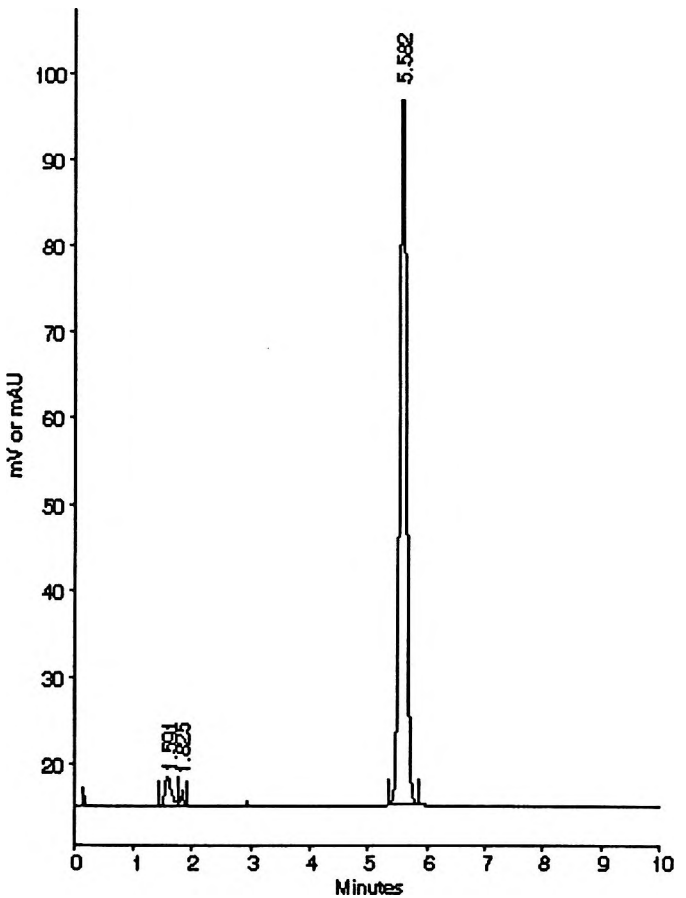



**Figure 7.** Varisoft SDC-85 Standard.

## Sample Preparation

Originally, the sample formulation was dissolved in mobile phase, filtered and injected. Due to the polyoxypropylene/polyoxyethylene fatty alcohols, the chromatograms were quite complicated. Interference from coeluted peaks gave inconsistent results. The sample preparation employed was a novel, simple means of selectively extracting quaternary ammonium compounds from fatty alcohols, oils and waxes.

### Precision of Recovery

The precision of recovery for the quaternary ammonium compounds was determined by assaying 10 separate sample preparations containing known concentrations of Varisoft 432 PPG (quat). The RSD of this experiment was 3.8%. The method accuracy was determined by comparing results obtained from the disclosed HPLC method to those obtained from gravimetric titration using silver nitrate. These results were in statistical agreement.

### Stress Study

Hair relaxer formulation prepared with Varisoft SDC-85 was stressed with heat (105°C, the pH of the formulation was 13) for 5 days. Five replicate samples were prepared following the sample preparation procedure with the exception that these samples were not left overnight. A degradation of over 60% was noted in all replicates. No interfering peaks at the retention times of the quat were observed.

## CONCLUSION

The described ion-exchange HPLC method for the quantitative determination of quaternary alkylammonium compounds in hair relaxer formulations is accurate, precise and reproducible. Additionally, the method is stability-indicating for alkylammonium quats at high pH.

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# NON-LINEAR DATA BUNCH

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## ABSTRACT

Application of data bunching to improve chromatographic quality can generally be performed in two ways. One approach is to apply linear data bunching which results in averaging data points and, consequently, risking the loss of information. By appropriately varying data bunch rates at opportune areas in a data series, the original chromatographic information will be preserved. This paper discusses the theory behind the development of a computer algorithm that automatically applies non-linear data bunching to chromatograms. Results show the reduction of data file size while improving chromatogram quality when compared to linear data bunch techniques.

## INTRODUCTION

Data bunching is a widely used technique in analytical chemistry to improve chromatographic or spectral peak slope and reduce random noise.[1] If during data bunching there is no loss of significant information, the technique can be used for data reduction. This will not only improve the quality of the chromatogram (or spectrum), but also increase data access by reducing data file size.

The "linear" data bunching method averages a fixed number of data points throughout a curve. For example, a 3 point linear data bunch means that groups of three data points are averaged. The application of data bunching is user selectable and can be applied to a whole or a part of a curve. Data bunching at an inappropriate rate can degrade the quality of a curve. The solution is to bunch data at different rates depending upon the importance of data at any one area.

Our goal is to bunch data automatically at a non-linear rate in order to preserve information, reduce data set size, improve integration accuracy, and eliminate user errors in the selection of a data bunch rate.

## EXPERIMENTAL

The example HPLC chromatogram was obtained on a Beckman System Gold (126 pump, 168 diode array detector) using software v 5.1, controlled by a Compaq ProLinea 4/50 computer.

The algorithm was developed with a Macintosh computer in HyperTalk, running under HyperCard.

## DISCUSSION

A linear data bunch averages a consecutive group of data points, regardless of their position in a chromatogram or spectra. A common problem is in a 4 point linear data bunch where the first point is at the baseline and the remaining three points are up-slope. The linear data bunch will average these points and part of the information will be lost. The worst scenario involves a very sharp peak (as the 12 minute peak in Figure 1) where one of the former points is in the up-slope, the second at the apex and the third on the down-slope. Averaging will reduce peak intensity.

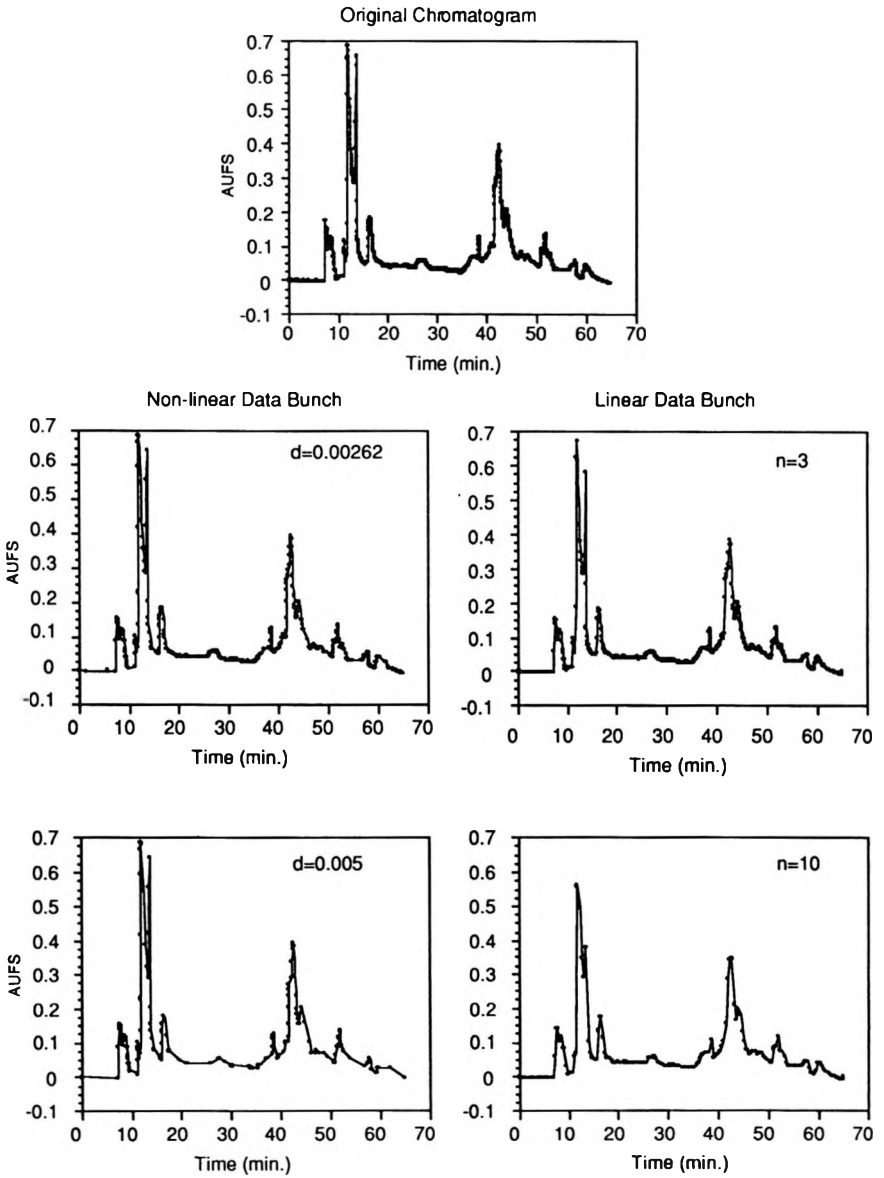


**Figure 1**. Non-linear and linear data bunch.

**Table 1**

**Data Sizes of Linear and Non-Linear Data Bunch Sets**

| Linear Bunch | | | Non-Linear Bunch | | |
|---|---|---|---|---|---|
| **Bunch By** | **No. of Data Points** | **% Red'n. in Size** | **d** | **No. of Data Points** | **% Red'n. in Size** |
| 0 pt | 1561 | 0.0 | 0 | 1561 | 0.0 |
| | | | 0.001 | 994 | 36.3 |
| 2 pt | 780 | 50.0 | 0.002 | 647 | 58.6 |
| 3 pt | 520 | 66.6 | 0.00262 | 475 | 69.6 |
| 4 pt | 390 | 75.0 | 0.003 | 408 | 73.9 |
| | | | 0.004 | 251 | 83.9 |
| 10 pt | 156 | 90.0 | 0.005 | 176 | 88.7 |

In the algorithm presented for non-linear data bunching, only data points that do not significantly change the slope are averaged. Thus, data points that carry information will be left intact. In this approach the data bunch rate for various parts of a curve is a function of the data itself. Such an approach can shrink data files up to 95% without significant loss of information. In contrast, a 10 point linear data bunch produces a file of the same size as a d = 0.005, but with significant information loss.

The non-linear data bunch algorithm was tested on a randomly chosen HPLC chromatogram and compared with linear data bunch results. The reduction of size is represented in Table 1. The "d" value represents the "tightness" of data compression. A lower value means that more data points will be left intact, and vice versa. The "d" value can be entered manually or calculated (estimated) using software and is constant throughout the chromatogram.

The d value of 0.00262 was estimated using our software. The original chromatogram had 1561 data points. The number of data points after non-linear data bunching depends on the information in the chromatogram. This is in contrast to linear data bunching which has a fixed number of data points regardless of the information the chromatogram carries.

Figure 1 represents the results of the calculation of linear and non-linear data bunch approaches with different "compression" ratios. Note that when the baseline is linear, it is represented with only four data points in a non-linear bunch (d= 0.00262) and the peaks contain most of the data points. The quality of the chromatogram is better than a comparable chromatogram with similar size applying linear data bunch (n= 3). With high data compression ratios (d = 0.005) there is also a noticeable baseline smoothing, without peak smoothing. However, in a linear data bunch chromatogram of similar size (n= 10) the data points are evenly distributed throughout the chromatogram. The loss of information is evident in linear data bunching with higher average rates. Note the loss of the fine structure of big peaks in a 10 point linear bunch and the loss of peak intensity (Figure 1 and Figure 2). The greatest data reduction is obtained in "clean" chromatograms, i.e., a straight baseline with a few sharp peaks; the data reduction approaches 98% with nc significant loss of information (data not shown).

## ALGORITHM

The algorithm[2] is basically a least squares fit. The set of equations describing a least squares fit[3] is:

$$y = ax + b$$

$$d = a + bx_i + y_i$$

$$a = \frac{\left(\sum y\right)\left(\sum x^2\right) - \left(\sum x\right)\left(\sum xy\right)}{n\sum x^2 - \left(\sum x\right)^2}$$

$$b = \frac{n\sum xy - \left(\sum x\right)\left(\sum y\right)}{n\sum x^2 - \left(\sum x\right)^2}$$

As a first step, the algorithm calculates a and b parameters in the equation y = ax+b through the first two points. Then, the line is extrapolated to the next data point and the $d_{n+1}$ value calculated. The d ($d_{n+1}$) value is defined by the equation that arises from a linear least squares fit and represents the distance between an extrapolated line and a data point (Figure 3). If the $d_{n+1}$ value is equal to or less than the estimated or entered d value, then the next point is added to the temporary list of data points and the calculation repeated. The d value determines how close the data points must be to the propagating vector.

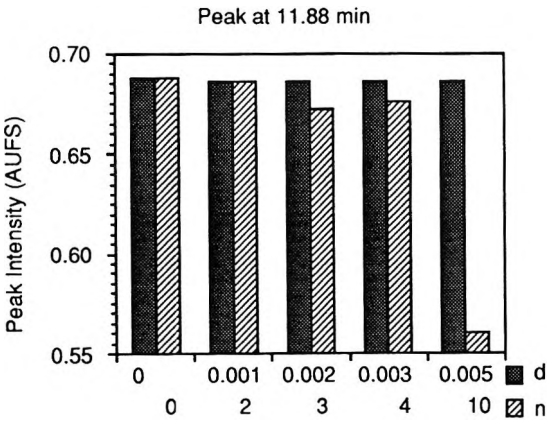


**Figure 2**. Reduction of peak intensity in non-linear and linear data bunch.

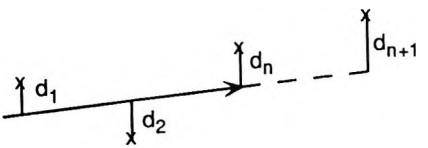


**Figure 3**. Least squares fit.

If the $d_{n+1}$ value exceeded the defined d value, then the data points in the temporary list of data points are averaged and stored in another data list that contains bunched data points. The pointer is then automatically shifted to the next data point and the process is repeated throughout the chromatogram or spectrum.

Since the algorithm is sensitive to the distance of the next point from the extrapolated line, it acts as a slope detector for the integrator routine. The difference is that the non-linear data bunch routine operates with a small slope detection limit.

## CONCLUSION

The algorithm for non-linear data bunching results in improved chromatogram quality. It also reduces the data file an average of 80% without

significant quality degradation. This algorithm can be applied to any type of spectra, e.g. NMR, UV, IR, etc. Such results can be obtained with a 13C NMR spectrum that contains a noisy baseline and very sharp peaks. Data compression in those instances is greater then 90% without data loss. This feature could be very useful with NMR spectra which have, in general, very large data sets.

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# REDUCTIVE VOLTAMMETRIC HPLC DETECTION OF AFLATOXINS: DETERMINATION OF AFLATOXIN $B_1$ IN FOODS

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## ABSTRACT

A reductive voltammetric detector, consisting of static mercury drop electrode was investigated for possible determination of aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ by HPLC. The HPLC system consisted of a $C_{18}$ ODS column and a mobile phase composed of 30% acetonitrile and 0.01M tetrabutylammonium bromide. The detector was operated in a differential pulse mode, set to the peak potential of -1.25 volts vs. Ag/AgCl. Under these conditions the sensitivity of the system was sufficient to determine aflatoxin $B_1$ present in several foods. Good agreement was obtained between this method and thin layer chromatography.

## INTRODUCTION

Aflatoxins are a group of toxic metabolites produced by certain strains of the common mold, Aspergillus flavus, as well as other mold genera. They were first found in peanut meal.

The levels of aflatoxins present in a number of foods was published by Wood[1] and Setsuko, et al.[2] Animal studies have shown most of them to be highly carcinogenic and, therefore, pose a health hazard to man.

Chemically, aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ are difurancoumarin derivatives. They are intensely fluorescent compounds, and consequently many methods use this property for their detection, such as in TLC and HPLC methods.[3] Problems exist, however, with the above methods in that TLC suffers from poor precision and HPLC from low specificity. Aflatoxins have been studied by oscillographic polarography[4] and by differential pulse polarography.[5] Their reduction potentials range from -1.21 to -1.27 volts (vs. SCE). These potentials are too close to determine individual aflatoxins when present in mixtures.

The purpose of this study was to investigate the possibility of applying reductive voltammetric detection in conjunction with the separation of some important aflatoxins by HPLC. The developed method could then be used for the analysis of food products.

## MATERIALS AND METHODS

### Apparatus

LC Pump: Waters Model 510 (Waters Assoc., Milford, MA); Injector: Rheodyne Model 17121 (Rheodyne Inc., Catati, CA); LC Column: Zorbax ODS, 5 μm, 4.6 mm x 25 cm (DuPont Co., Wilmington, DE); Guard Column: 7 cm x 2.1 mm id., packed with 25-37 μm Co:Pell ODS (Whatman, Inc., Clifton, NJ); Polarographic Analyzer: EG & G PAR Model 264A (EG & G Princeton Applied Research, Princeton, NJ); Static Mercury Drop Electrode: EG & G Model 303 (EG & G Princeton Applied Research, Princeton, NJ); Detector: EG & G PAR Model 310, (EG & G Princeton Applied Research, Princeton, NJ)

A schematic diagram of the apparatus is shown in Figure 1.

### Reagents

Aflatoxin Standards: Individual $B_1$, $B_2$, $G_1$, and $G_2$ aflatoxin standards received as dry films were separately diluted to nominal concentrations of 10 μg/mL with benzene-acetonitrile (98+2). The standard concentrations were

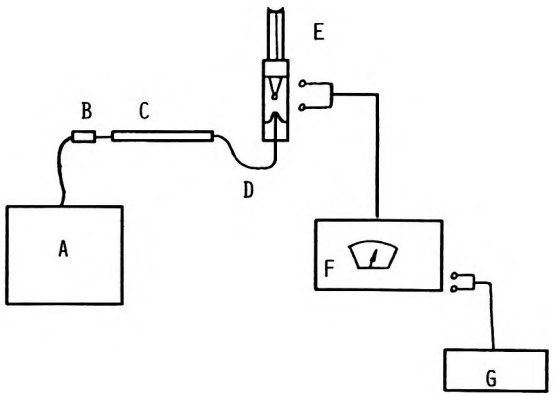


**Figure 1**. Schematic diagram of apparatus. A, HPLC pump; B, Guard column; C, HPLC column; D, Eluate; E, Mercury drop electrode; F, Polarograph; G, Strip chart recorder.

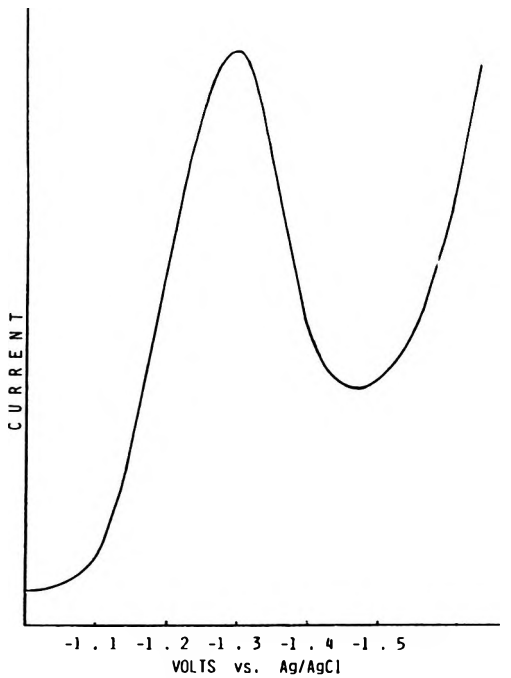


**Figure 2**. Differential pulse polarogram of a mixture of aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ in the mobile phase. Polarographic conditions: pulse amplitude, 100 mv; pulse time, 0.5 s; electrode, hanging mercury drop; sensitivity setting, 10 μA full scale.

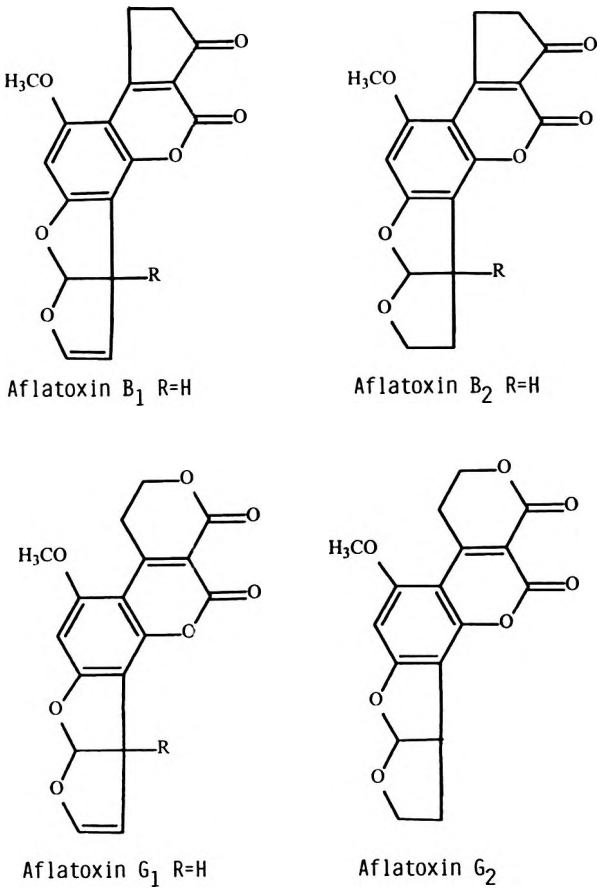


**Figure 3**. Structural formulas of the four aflatoxins.

determined spectrophotometrically using $K_2Cr_2O_7$ primary standard to calibrate the spectrophotometer (AOAC, 1990). For analysis by the proposed HPLC method the benzene-acetonitrile was evaporated at low heat in a current of nitrogen. The residue was then dissolved in the mobile phase.

Mobile Phase: 30% acetonitrile and 0.01M tetrabutylammonium bromide. Oxygen was removed by purging with nitrogen for twenty minutes, then nitrogen was directed above the mobile phase during analysis to prevent oxygen from re-entering.

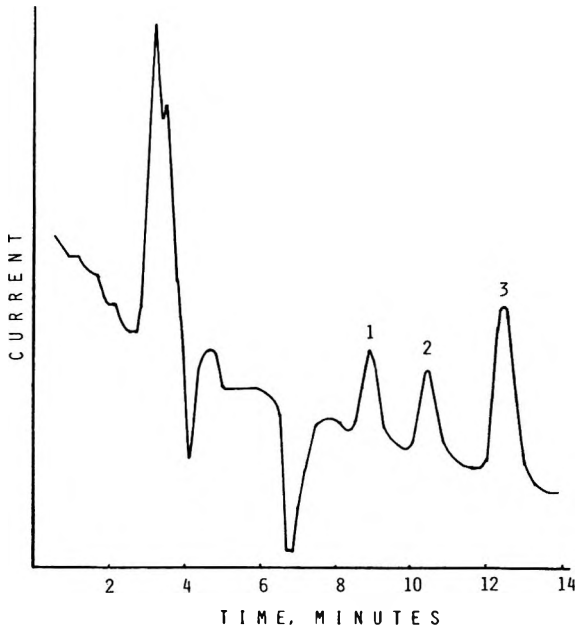


**Figure 4**. Chromatogram of a mixture of aflatoxins. Peak 1, $G_2$; Peak 2, $B_2$+$G_1$; Peak 3, $B_1$.

**Table 1**

**Results of Sample Analysis by TLC and Proposed HPLC Methods**

**Found, ng/g[1]**

| | TLC | | | | HPLC | | | |
|---|---|---|---|---|---|---|---|---|
| | **B1** | **B2** | **G1** | **G2** | **B1** | **B2** | **G1** | **G2** |
| Melon seeds | 38 | 4 | 0 | 0 | 43 | ND | ND | ND |
| Corn meal | 43 | 5 | 0 | 0 | 42 | ND | ND | ND |
| Corn flakes | 54 | 11 | 0 | 0 | 48 | ND | ND | ND |
| Mixed nuts | 0 | 0 | 0 | 0 | ND | ND | ND | ND |

[1] Single determinations.
ND: Not detected.

### Samples

Melon seeds, corn meal, corn flakes and mixed nuts were obtained as samples from the importers of these products.

### Preparation of Samples

Samples were prepared and analyzed according to the official CB method.[3] The extracts were contained in vials in small volumes of benzene-acetonitrile (98+2) and were stored in a freezer. Prior to the analysis by the proposed method, the solvent was evaporated at low temperature in a current of nitrogen. The residues were then dissolved in 0.5 mL of the mobile phase for analysis by the proposed HPLC method.

### Procedures

Polarography: Differential pulse polarograms were run on standards dissolved in the mobile phase in order to determine the potential setting required for the detection in HPLC effluents.

HPLC conditions: Flow rate, 1 mL/min.; potential, -1.25 volts vs. Ag/AgCl; electrode: hanging mercury drop; mode, differential pulse; modulation amplitude: 100 mv; volumes injected: 200 μL.

## RESULTS AND DISCUSSION

Prior to HPLC analysis, it was necessary to study the voltammetric behavior of the compounds in question. A mixture of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ dissolved in the mobile phase of 30% acetonitrile-0.01M tetrabutylammonium bromide and polarographed in a differential pulse mode gave a single peak at -1.25 volts vs. Ag/AgCl (Fig. 2). This was to be expected due to the similarity of their chemical structures, Wilson et al.,[6] (Fig. 3). Solutions of individual aflatoxins and their mixtures were injected into the proposed HPLC system in order to obtain their retention times, separation and detection parameters. A chromatogram of a mixture of the four aflatoxins is shown in Fig. 4. Peaks 1 and 3 are aflatoxins $G_2$ and $B_1$, respectively, while peak 2 represents unseparated aflatoxins $B_2$ and $G_1$. No attempt was made to separate these aflatoxins at the present time since the primary objective of this study was to determine the feasibility of voltammetric detection.

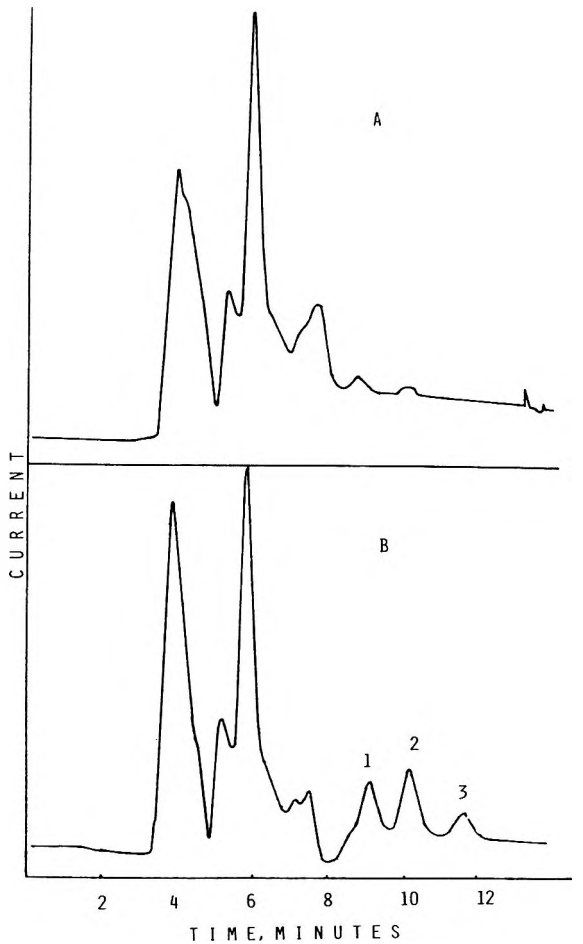


**Figure 5**. Chromatogram of a mixed nut sample, A; and B, mixed nut sample spiked with 20 ng/g $B_1$, $B_2$, $G_1$, and $G_2$. Peak 1, $G_2$; Peak 2, $B_2$+$G_1$; Peak 3, $B_1$.

In order to determine the practicality of the technique, several different sample extracts and spikes were injected into the proposed chromatographic system. Typical chromatograms of a nut sample and spike are shown in Fig. 5, while a chromatogram of a corn sample is shown in Fig. 6. Since the retention times were somewhat affected by the sample matrix, identification of the peaks was made by standard additions. In order to compare the proposed method with the official TLC method, experiments were conducted on the food samples.

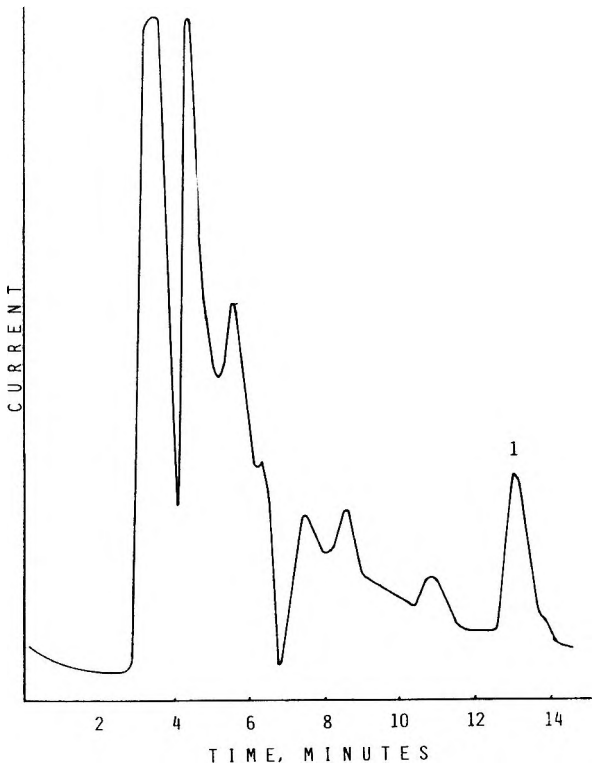


**Figure 6**. Chromatogram of corn sample. Peak 1, aflatoxin $B_1$.

The results of this comparative test are given in Table 1. The sensitivity of the proposed HPLC method was sufficient only to detect and quantitate aflatoxin $B_1$; the detection limit was 2.5 ng/g. The agreement for this aflatoxin between the methods, however, is very good.

## CONCLUSION

The method as tested was able to determine aflatoxin $B_1$ which may occur in foods. It should be useful for confirmation of results found by the TLC method. In order to detect other aflatoxins that may be present at very low levels (<ppb to low ppb) it will be necessary to modify the HPLC conditions and enhance the sensitivity of the system.

A possible approach may be to increase the sample size and to switch to a gold/mercury electrode connected to a polarographic analyzer capable of differential pulse mode of detection. Work is continuing in this area.

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# SOLID-PHASE EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMITRIPTYLINE AND NORTRIPTYLINE IN HUMAN PLASMA

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## ABSTRACT

A simple, rapid, cost-efficient solid-phase extraction and high performance liquid chromatographic method for the determination of amitriptyline and nortriptyline in human plasma is described. After conditioning of solid-phase sorbent diluted plasma samples were passed through the cartridge. Potentially interfering substances were washed, followed by elution of amitriptyline, nortriptyline and maprotiline (internal standard) from the sorbent. Eluates were collected, evaporated, reconstituted and injected directly and monitored at 215 nm. Samples were chromatographed on a 5 μm Supelcosil LC-PCN (150 x 4.6 mm) using 0.01 M dipotassium hydrogenphosphate (pH adjusted to 7 with 85% ortophosphoric acid)/ acetonitrile/

methanol (15:60:25, v/v) as the mobile phase. Recoveries for amitriptyline and nortriptyline were 96% and 85%, respectively. The limit of detection and quantification for amitriptyline and nortriptyline were 1 ng/mL and 5 ng/mL, respectively.

The calibration curves for amitriptyline and nortriptyline in human plasma were linear over the range 5-500 ng/mL. The method was applied in single dose (75 mg) clinical pharmacokinetic studies of amitriptyline in depressed patients.

## INTRODUCTION

Tricyclic antidepressants continue to be one of the most widely used classes of psychotropic drugs employed in clinical practice on a long-term basis. In 1985 the American Psychiatric Association Task Force recommended therapeutic monitoring of tricyclic antidepressant drugs as a guideline for treating patients.[1] To achieve this goal various methods for the determination of tricyclic antidepressants in plasma have been developed and reviewed.[2-4]

Amitriptyline (AT) is the most widely used tricyclic antidepressant. Its main metabolite, nortriptyline (NT), formed by mono-N-demethylation, also contributes to the pharmacological activity and is itself marketed as a therapeutic agent.

Various methods have been described to determine the concentration of AT and NT in plasma including radioimmunoassay,[5] gas chromatography,[6,7] and high performance liquid chromatography-HPLC.[8-22] Gas chromatographic determination of AT is specific and sensitive but requires lengthy derivatization steps of the secondary amine formed. Among various analytical techniques used to measure AT and NT plasma levels in recent years, HPLC appears to be the most suitable for routine laboratory needs due to its versatility, availability, and reliability. Among these methods some are based on normal phase[9,11,13,18] or reversed phase,[8,14-17,19,20] and ion-pair reversed phase.[10,12]

The previous isolation techniques for AT and NT from plasma were generally accomplished by liquid-liquid extraction of an aqueous sample into an organic solvent, followed by back-extraction of the organic layer into another aqueous phase. The organic solvent is then, evaporated to dryness before reconstitution in the appropriate solvent. However, these methods are time-consuming and labor-intensive.

Therefore, the present paper will focus on simple, rapid, cost-efficient solid-phase extraction followed by a specific and accurate liquid chromatographic method which could be applied on a single dose clinical pharmacokinetic study of AT in depressed patients.

## MATERIALS AND METHODS

### Reagents and Chemicals

AT hydrochloride and NT hydrochloride were obtained from Lundbeck Co. (Amsterdam, The Netherlands), maprotiline hydrochloride was a gift from Pliva (Zagreb, Croatia). Ortophosphoric acid, dipotassium hydrogenphosphate and ammonium hydroxide (analytical grade) were obtained from Merck (Darmstadt, Germany). Methanol and acetonitrile HPLC grade (Licrosolv$^{R}$) were purchased from Merck (Darmstadt, Germany).

### Apparatus

HPLC system Hewlett Packard (Avondale, PA, USA) model 1050 was used, equipped with a diode-array UV detector Hewlett Packard and an integrator model HP 3396A (Hewlett Packard, Avondale, PA, USA). A model 7125 sample injector and six-port switching valve (Rheodyne, Cotati, CA, USA) were used. The guard column (20 x 4.6 mm) packed with 3 μm Supelguard LC-CN (Supelco, Bellefonte, PA, USA) and analytical column (150 x 4.6 mm) packed with 5 μm Supelcosil LC-PCN (Supelco, Bellefonte, PA, USA) were used.

For sample preparation, we used 1 mL Supelclean LC-WCX SPE tubes and VISI$^{TM}$-1 Single SPE tube processor.

### Chromatographic Conditions

The mobile phase consisted of 0.01 M dipotassium hydrogenphosphate (pH adjusted to 7 with 85 % ortophosphoric acid)/acetonitrile/methanol (15:60:25, v/v). A flow rate of 2 mL/min was used at ambient temperature. The mobile phase, after mixing, was filtered through a 0.45 μm membrane filter by vacuum and degassed in an ultrasonic bath prior to use. Analysis was performed at 215 nm.

**Standards for Calibration Graphs**

AT hydrochloride (0.00226 g) and NT hydrochloride (0.00227 g) were weighed out exactly and dissolved in a 100 mL volumetric flask with mobile phase to give final concentration of 20 mg/L. Working solutions were prepared by appropriate dilutions of the stock solution with mobile phase to give final concentrations of 5, 25, 50, 100, 200, 400 and 500 ng/mL AT and NT. Plasma standards for calibration curves were prepared by spiking 1 mL aliquots of pooled drug free plasma with appropriate volumes of the above mentioned working solutions to make AT and NT plasma standards ranging from 5 to 500 mg/L . Internal standard - maprotiline (MT) (10 μL of 20 mg/L) was added in plasma standards. Calibration graphs of the recovered standards were prepared for each day of analysis to establish linearity and reproducibility of the HPLC system. Calibration curves were obtained by plotting the ratio of peak area of AT and NT to internal standard against drug concentrations.

**Sample Preparation**

AT and NT were isolated from human plasma by solid-phase extraction with 1 mL Supelclean LC-WCX SPE tubes. The solid-phase sorbent was conditioned with 500 μL of 0.5 M ortophosphoric acid followed by 1 mL of deionized water. 500 μL of plasma samples containing 10 μL of MT (20 mg/L) mixed with 500 μL of deionized water, then, passed through the sorbent. Analytes (AT, NT and MT) were bound onto the sorbent, and interfering substances were washed with an appropriate solvent system (1 mL of deionized water, 500 μL of 1 M ammonium hydroxide in water, twice with 1 mL of 5 % methanol in water). Analytes were , then eluted on-line from the sorbent twice with 1 mL of 0.22 M ammonium hydroxide in methanol.

The eluate was collected in silanized glass vial, evaporated to dryness under nitrogen and reconstituted in 250 μL of HPLC mobile phase. Throughout the procedure silanized glassware was used, due to irreversible adsorption of AT and NT by untreated borosilicate glass.

**Analytical Variables**

Extraction efficiency was studied by adding known amounts of AT and NT to drug free plasma. After extraction and injection into the chromatograph, the ratio of peak area of AT and NT to MT obtained was compared with the

corresponding ratio obtained with standard solution of AT and NT in mobile phase. The precision and accuracy of the method were evaluated by repetitive analysis of plasma spiked with AT and NT.

### Analysis of Plasma Samples

The HPLC procedure was used to study clinical pharmacokinetics of AT and NT in adult depressive patients on AT monotherapy. AT and NT plasma concentrations were determined after single dose administration of 75 mg of AT. Blood samples (5 mL) were withdrawn just before drug administration (0 hr) and at 1, 2, 3, 4, 6, 9, 12, 24, 36, and 48 hr after single oral dose. At the steady state (on the 14 th day of therapy) five blood samples were obtained.

## RESULTS

Under the chromatographic conditions described, the retention times for AT, NT and MT were 3.7 , 7.5 and 10.5 minutes, respectively. Figure 1A shows the chromatogram obtained from an extracted drug free plasma. No peaks corresponding to the drug, its metabolite or internal standard retention times, were found. Figure 1 B shows the chromatogram obtained from extracted plasma of a patient receiving AT monotherapy. Good selectivity of the method was indicated by the lack of interfering peaks in plasma.

### Validation Of The Method

### Recovery

Extraction recovery data from plasma samples spiked with known amounts of AT and NT are presented in Table 1.

### Linearity And Sensitivity

A typical calibration curves of AT and NT from plasma were linear in the range of 5-500 ng/mL.

The average regression equation and regression coefficient for AT were $y= 0.0056 x + 0.043$ (where y= peak area ratio of AT to MT and x= plasma concentration of AT ) and $r= 0.9996$, respectively.

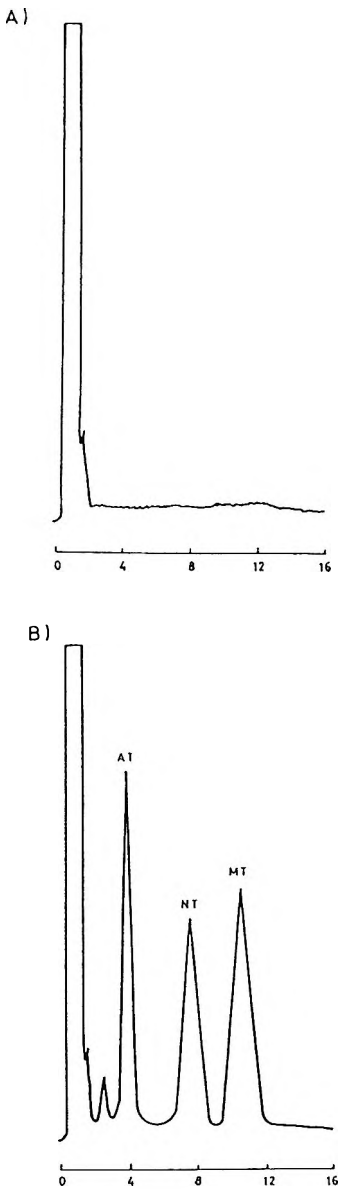


**Figure 1**. Chromatograms of extracted drug free plasma A) and extracted plasma of a patient on AT therapy B).

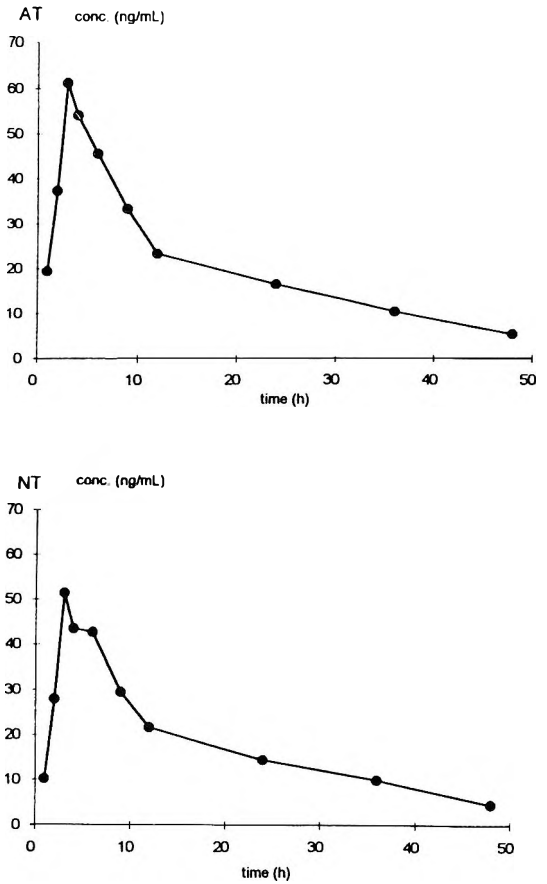


**Figure 2**. Mean plasma concentrations of AT and NT B) in 9 depressed patients on AT monotherapy after single dose adminsitraiton (D = 75 mg).

The average regression equation and regression coefficient for NT were y=0.0057 x + 0.11 (where y= peak area ratio of NT to MT and x=plasma concentration of NT ) and r=0.9994, respectively.

The limit of detection, arbitrarily defined as 5 times baseline noise, was 1 μg/L for AT and NT while the limit of quantification was 5 μg/L for both compounds.

**Table 1**

**Recoveries of AT and NT from Spiked Plasma Samples (N = 5)**

| Cond. Added (ng/mL) | Conc Found (ng/mL) | | Recovery (%) | |
|---|---|---|---|---|
| **AT/NT** | **AT** | **NT** | **AT** | **NT** |
| 5 | 4.75 | 4.25 | 95 | 85 |
| 25 | 24.08 | 22.10 | 96 | 88 |
| 50 | 49.11 | 44.54 | 98 | 89 |
| 100 | 96.13 | 86.73 | 96 | 87 |
| 200 | 188.53 | 164.13 | 94 | 82 |
| 400 | 392.14 | 328.63 | 98 | 82 |
| 500 | 465.34 | 405.12 | 93 | 81 |
| | | **mean:** | 96 | 85 |
| | | **+/-SD:** | 2 | 3 |

**Table 2**

**Within-run (CV) and Day-to-day (CV*) Coefficient of Variation of AT and NT from Spiked Plasma Samples (N = 5)**

| Conc. (ng/mL) | CV(%) | | CV* (%) | |
|---|---|---|---|---|
| **AT/NT** | **AT** | **NT** | **AT** | **NT** |
| 5 | 7.1 | 6.3 | 8.2 | 8.8 |
| 25 | 4.3 | 8.1 | 6.1 | 9.2 |
| 50 | 3.7 | 7.1 | 4.1 | 8.2 |
| 100 | 2.6 | 4.1 | 3.9 | 5.6 |
| 200 | 5.1 | 6.3 | 4.3 | 5.8 |
| 400 | 6.2 | 7.7 | 5.1 | 6.6 |
| 500 | 7.1 | 7.9 | 6.2 | 6.8 |
| **mean:** | 5.2 | 6.8 | 5.4 | 7.3 |
| **+/- SD:** | 1.7 | 1.4 | 1.5 | 1.4 |

### Accuracy and Precision

The accuracy of the method is indicated by the error of assayed samples relative to their spiked concentrations. The method demonstrated superior accuracy in that all errors were less than 10 %. The method was also shown to be precise in studies of replicate assays. Study demonstrated coefficient of variation within-run (CV) and day-to-day (CV*) less than 10 % which demonstrates good reproducibility of the method (Table 2).

### Application

The mean plasma concentration-time curves of AT and NT obtained after oral administration of a single dose of 75 mg of AT (N=9) in depressed patients are presented in Figure 2.

## DISCUSSION

Because large variations in the steady state concentration of AT can occur in patients receiving the same oral dose,[23] successful therapy using AT depends upon the reliable monitoring of plasma levels.[24] The analysis of AT (and NT) from plasma has been accomplished using various analytical procedures including radioimmunoassay, gas chromatography, HPLC based on normal phase, reversed phase, and ion-pair reverse phase.

Previous extraction techniques for AT have been time-consuming and labor-intensive. For routine investigations a simple and rapid sample preparation procedure with a reproducible and effective extraction of AT and NT is desirable.

In recent years, investigators have demonstrated the merits of solid-phase sorbent extractions over conventional liquid-liquid techniques for drug extractions. Cleaner extracts, lower solvent consumption, and reduced analysis times have been reported advantages.

In the current method, solid-phase extraction is used. After conditioning of solid-phase sorbent diluted plasma samples were passed through the cartridge. Potentially interfering substances were washed, followed by elution of AT, NT and MT from sorbent. To ensure optimum drug recovery a slow dropwise flow rate was maintained during the sample addition and sample elution steps of the extraction procedure. Rapid flow rates reduced recovery for 15-25%. Dilution of the sample with deionized water prior to extraction, by

reducing the concentration and ionic strength of unwanted plasma components, allowed them to pass more easily through the tube. More concentrated solutions of ammonium hydroxide used for packing wash. or larger wash volumes would remove AT and NT from packing, reducing drug recovery for 20-30 %. Due to irreversible adsorption of AT and NT by untreated borosilicate glass we used silanized glassware throughout the procedure.

The current method was found to reduce errors due to sample manipulation, which provided improved reproducibility as well as superior recovery. The sorbent extraction is therefore more convenient, and much less time is required for sample processing. The saving in the time for the analysis using solid-phase extraction is the most important favorable factor for this method. Since elution solvent volume is low, evaporations presents no problem, and selective elution procedures yield final samples with fewer endogenous impurities than are usually obtained in liquid-liquid extraction methods.

Also the chromatographic system provided a fast establishment of equilibrium between the mobile and the stationary phase, a stable column performance even after prolonged usage, as well as a relatively short run time for assay.

Small sample volume (0.5 mL) combined with simple, rapid, cost-efficient solid-phase extraction, short chromatographic time and minimal source of interference has been achieved in the method described here. The combination of obtained features (the simplicity, selectivity, sensitivity and cost effectiveness of solid-phase extraction) has allowed the development of a method that is accurate, precise, sensitive, and ideally suited for monitoring therapeutic and/or toxic levels of AT and NT. The method presented in this paper has also proved to be useful in single-dose clinical pharmacokinetic studies of AT in depressed patients.

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# QUALITATIVE AND QUANTITATIVE DETERMINATION OF METHYL ESTERS, FREE FATTY ACIDS, MONO-, DI-, AND TRIACYLGLYCEROLS VIA HPLC COUPLED WITH A FLAME IONIZATION DETECTOR

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## ABSTRACT

High performance liquid chromatography (HPLC) with a cyanopropyl phase column coupled directly to a commercial HPLC flame ionization detector (FID) and a gradient mobile phase of tert-butyl ether and hexane proved successful to separate and quantitate mono-, di-, and triacylglycerols, free fatty acids and methyl esters. These components can occur together during glycerolysis, lipolysis, randomization, and interesterification reactions of vegetable oils like soybean. Gravimetric standards were evaluated by HPLC-FID in which each contained mono-, di, and triacylglyceols, free fatty acids and methyl esters of palmitic, stearic, oleic, linoleic, and linolenic acids respectively. The FID response with solute weight decreased in this order: triacylglycerols, free fatty acids, diacylglycerols, methyl esters, and monoacylglycerols, respectively. The FID thus required response factors to quantitate the components of mixtures of diverse lipid species. However, for homogenous compounds the

FID response was linear and response factors were not required, for example, for a series of free fatty acids like palmitic through linolenic. The commercial HPLC-FID proved satisfactory for facile quantitation of diverse lipid species in vegetable oil reaction mixtures. Good accuracy was obtained by HPLC with FID for soybean glycerolysis products, which contained mono, di and triacylglycerols.

## INTRODUCTION

Interesterification is a process used in the oils and fats industry to modify the properties of triacylglycerols (TAG). This reaction is of commercial interest for the production of margarines, shortenings, and other speciality products with desired physical properties and oxidative stability.[1-4] Depending on interesterification conditions, the side reactions of lipolysis and hydrolysis may occur.[5-7] Other reactions, such as glycerolysis to produce products like monoacylglycerols (MAG) and diacylglycerols (DAG) from vegetable oil TAG are important in the food industry. Here too, it is important to monitor the occurrence of free fatty acids (FFA), MAG, DAG, and unreacted TAG.[8] Other important food industry reactions, which require monitoring of diverse lipid species (LS) of FFA, MAG, DAG, and TAG, include vegetable oil TAG hydrolysis to produce industrially or nutritionally important FFA.[9,10]

Analytical methods like thin layer (TLC)[11,12] and gas chromatography (GC)[13] and high performance liquid chromatography (HPLC)[14-21] have been used to analyze LS. TLC with FID and silica gel coated rods for LS was reviewed by Sebedio.[11] He pointed out that the GC FID response was not a linear relationship with sample amount spotted on these rods. Even for homogeneous compounds, a power function was necessary to describe FID response with respect to sample amount spotted on the TLC rod. Also, recently, Peyrou reported for TLC of oleic acid LS, the TLC-FID required power functions for calibration curves to express detector response with amount of LS spotted on the TLC rods.[12] TLC-FID response is usually linear without the need for response factors for homogeneous components of lipid mixtures.[13] However, response factors are required for GC analysis of mixtures of diverse LS. Also, to avoid thermal alteration at the elevated temperature required for GC, LS like MAG and DAG required derivatization before analysis.[13] HPLC, which does not require elevated temperature, avoids the need for derivatization and has been reported as the method of choice for LS.[13-21] Unfortunely, for HPLC of LS, detection is a problem.[14-19] Presently, the light scattering and flame ionization detectors show the most promise in LS analysis.[14-19] However,

light scattering detector response is not a linear relationship with solute amount and complex calibration curves are required for quantitation.[17,19] On-the other-hand, transport flame ionization detectors of the moving wire type[14] or rotating quartz belt[15,17] have been reported to demonstrate a linear response with solute amount without the need for response factors for homogenous LS.[14-20] We have previously described many examples of quantitative reversed phase HPLC coupled with a commercial HPLC-FID with a rotating quartz belt for TAG in vegetable oils, vegetable oil blends and products.[1-4,20] However, the rotating quartz belt FID response has not been evaluated for mixtures in which diverse LS like MAG, DAG, TAG, FFA, and methyl esters (FAME) occur together.[17]

This study describes the resolution and quantitation of identified, diverse LS with a cyanopropyl bonded phase HPLC column coupled with a commercial HPLC-FID of the rotating quartz belt design.

## EXPERIMENTAL†

### Materials

Lipid standards were purchased from Nu Chek Prep, Inc.(Elysian, MN) and Sigma Chemical Co . (St. Louis, MO). All solvents were HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ) and J T. Baker Inc. (Phillipsburg, NJ).

### High Performance Liquid Chromatography

HPLC was performed with a Thermo Separation Products (Schaumburg, IL) (Model SP 8800) ternary solvent system with a HPLC column of bonded cyanopropyl phase, Alltech Associates (Deerfield, IL) Econosil CN. 25 cm x 4.6 mm, 5 μm. A gradient solvent program with 0.5% acetic acid (AA) in tert-butyl ether (TBE) and hexane (HEX), modified from that used by El-Hamdy and Christie[21] was used to accomplish the LS separation as follows: 2% (0.5% AA in TBE) in HEX (V:V ) for 6 min., linear from 6-34 min to 100% (0.5% AA in TBE) hold 10 min then return to initial conditions 44-54 min. The flow rate was 1.0 mL/min. The 0.5% AA in TBE was required to prevent FFA being adsorbed by the HPLC column. The sample size range was 0.063 to 0.5 mg in 10 μL 0.5% AA in TBE. The HPLC detector was a commercial HPLC-FID with a moving quartz belt, Finnigan, Inc. (Austin, TX) Tremetrics Model 945. The HPLC-FID was operated with block temperature 130°C; detector gas, 140 mL/min. hydrogen; cleaning flame, 275 mL/min hydrogen;

175 mL/min oxygen; and compressed air, 0.4 cubic ft/min. The FID response was processed by a real-time computer.[22] Identification and quantitation of the HPLC-FID was based on known gravimetric mixtures of FAME; TAG; FFA, 1,2-and 1,3-DAG. and 1- and 2-MAG.

## RESULTS AND DISCUSSION

A technique reported by El-Hamdy and Christie[21] for HPLC of an unidentified mixture of LS on a cyanopropyl bonded phase column gave stable retention times, resolution and symmetrical chromatogram peaks for the mixture of TAG, FAME, MAG, DAG, and FFA. These workers used a light scattering detector and did not report quantitation for the LS. HPLC detectors like those based on light scattering and ultraviolet absorption principles require complex calibration curves for quantitation.[17]

We extended the cyanopropyl column resolution to more mixtures of diverse LS, which are identified. Further, we used a commercial HPLC-FID of the moving quartz belt type for quantitative analysis of LS in this study. This HPLC-FID utilized the following operational procedures: 1) the HPLC effluent is sprayed onto a rotating quartz belt, 2) the HPLC solvent is removed by high temperature and partial vacuum, 3) solutes remain on the belt, which is rotated through a FID to produce a response proportional to solute quantity, and 4) the belt is passed through a cleaning flame and returned to the starting position. There is no interface between the HPLC column exit and the detector. Previously, Christie presented a schematic of the detector.[15] The detector response is reported linear with solute concentration and does not require complex calibration curves for quantitation.[15,17]

Separation on the cyanopropyl column of LS gravimetric standards or series and reaction mixtures of FAME, TAG, FFA, DAG, and MAG is presented in Figure 1. The series for LS, which contain stearic, palmitic, oleic, linoleic, and linolenic acids exclusively, are arranged sequentially starting at the figure bottom. It is observed that the elution times for the components on the cyanopropyl column increased in this order: FAME; TAG; FFA; 1,3-DAG;1,2-DAG; 1-MAG and 2-MAG respectively. Retention time precision was an average of plus or minus 0.50 min. per LS component for triplicate analysis. Also, retention times were stable and reproducible over the two month period the cyanopropyl column was used. The LS components of the stearic acid series have the earliest retention times. The retention times for the other LS components increased in this order: palmitic, oleic, linoleic, and linolenic as the FA moiety polarity increased.

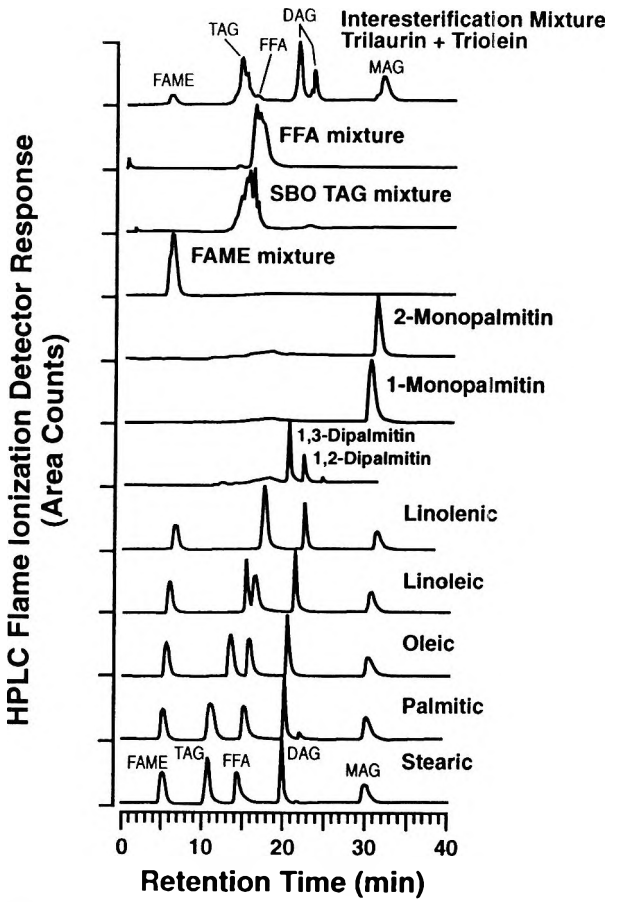


**Figure 1**. HPLC separation of lipid compounds with an acetic acid/methyl tert-butyl ether/hexane gradient on a cyanopropyl column coupled with an HPLC flame ionization detector.

The retention time changes are slight and in the direction expected for polar HPLC. For the LS linolenic series the TAG and FFA co-elute. Also, in Figure 1, it is observed that 1,2- and 1,3-palmitic DAG are resolved and 1 and 2 palmitic MAG are partially resolved. As, expected, the less polar of the DAG and MAG pairs eluted first. It is observed during HPLC, little DAG or MAG isomerization occurred even though the mobile phase contained acetic acid. Compared to the gravimetric standard series and model DAG and MAG, which

**Table 1**

**HPLC-FID Quantitation of Lipid Species**

| Gravimetric Mixture | Components | HPLC-FID Area %[a] | Weight % | Absolute Error%[b] |
|---|---|---|---|---|
| Stearic | FAME | 18.3 | 20.0 | 1.7 |
| | TAG | 25.5 | 20.0 | 5.5 |
| | FFA | 20.8 | 20.0 | 0.8 |
| | DAG | 19.9 | 20.0 | 0.1 |
| | MAG | 15.5 | 20.0 | 4.5 |
| | | | Average error | 2.5 |
| Oleic | FAME | 17.9 | 20.0 | 1.8 |
| | TAG | 23.5 | 20.0 | 3.8 |
| | FFA | 21.7 | 20.0 | 0.5 |
| | DAG | 21.1 | 20.0 | 1.4 |
| | MAG | 15.8 | 20.0 | 3.9 |
| | | | Average error | 2.3 |

[a] Area % precision, standard deviation = ± 0.0 to 0.5% triplicate analysis.[23]

[b] Difference between experiment area % and weight %.

gave single chromatogram peaks for each LS component, FAME (FA14:0,16:0,18:0,18:1,18:3,20:0) FFA (FA 16:0,18:0,18:1,18:2,18:3) and SBO TAG (trilinolein-mixed FA TAG-tristearin) and mixtures of DAG and MAG from an interestification reaction of trilaurin and tristearin gave somewhat broad peaks. However, these latter mixtures have LS component elution in the appropiate FAME, TAG, FFA, DAG, and MAG regions of the chromatogram to allow identification of these respected LS in samples from vegetable oil interesterification, glycerolysis, or hydrolysis reaction mixtures.

Plots of HPLC-FID response for each of the LS components in the gravimetric standards for component weight of 0.06 to 0.1 mg were linear with linear regression coefficients[23] range of 0.9916-0.9999. The FID response with component weight decreased in this order:TAG, FFA, DAG, FAME and MAG, respectively. The magnitude of the detector response per component weight or

**Table 2**

**HPLC-FID Responce Factors[a,b] for Lipid Species**

| Gravimentric Mixture | Components | Response Factors |
|---|---|---|
| Stearic | FAME | 1.09 |
| | TAG | 0.78 |
| | FFA | 0.96 |
| | DAG | 1.00 |
| | MAG | 1.29 |
| Oleic | FAME | 1.12 |
| | TAG | 0.85 |
| | FFA | 0.92 |
| | DAG | 0.95 |
| | MAG | 1.26 |

[a] Response factor = gravimentric component wt% ÷ HPLC=FID area %.

[b] Response factor precision ± 0.05 for triplicate analysis.

response factors[24] ranged from 4.10 for TAG to 2.90 for MAG ($x10^6$ area counts per mg sample). These FID response results with respect to LS type, i.e., TAG vs. MAG, are similar to the concept of relative weight proportion of FID active carbons.[25-27] For example, MAG gave a lower detector response than TAG since MAG has fewer active carbons (methylene carbons) which are responsible for FID response.

Once linearity of detector response was established for the components of the LS gravimetric standards, the HPLC-FID quantitation was examined. Samples of palmitic, stearic, oleic, linoleic, and linolenic gravimetric series (Figure 1) with FAME, TAG, FFA, TAG, and MAG each 20% by weight were injected onto the cyanopropyl HPLC column and the FID area percent obtained by computer integration of the FID response.[22] The FID area percent and gravimetric weight percent (20 %) are compared for the LS of the stearic and linoleic gravimetric standards in Table 1. It is observed that the HPLC-FID area percent is within 1 percent of the weight percent for FFA. For FAME and DAG, the FID area percent is within 2 percent of the weight percent.

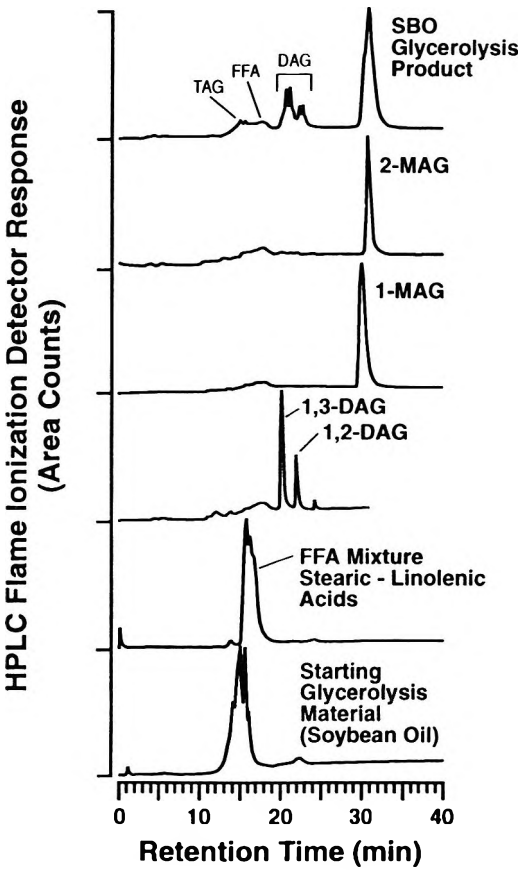


**Figure 2.** HPLC separation of a soybean oil glycerolysis mixture with an acetic acid/methyl tert-butyl ether/hexane gradient on a cyanopropyl column coupled with an HPLC flame ionization detector.

The HPLC-FID area is 2 percent greater than weight percent for TAG and MAG. Similar results were obtained for palmitic, linoleic and linolenic series, with average errors over all the LS components of 3 percent.

The results in Table 1 show that for good quantitation with HPLC-FID for mixtures which contain FAME, TAG, FFA, DAG, and MAG, response factors are required. Response factors calculated by dividing gravimetric weight percent by HPLC-FID area percent[24] are given for the LS of the stearic and oleic series in Table 2.

**Table 3**

**HPLC-FID Analysis of Lipid Species in a Soybean Oil Glycerolysis Mixture[a]**

| Analytical Method | Compounds | Uncorrected Area % | Corrected[b] Area % |
|---|---|---|---|
| HPLC-FID | TAG | 1.01 | < 0.1 |
| | FFA | 1.6 | 2.0 |
| | DAG | 26.4 | 21.8 |
| | MAG | 71.1 | 76.2 |
| SCF[c] | TAG + FFA | | 1.5 |
| | DAG | | 23.3 |
| | MAG | | 75.3 |

[a] Chromatogram of glycerolysis reaction presented in Fig. 2.
[b] Uncorrected area % adjusted by average response factors given in text and the data renormalized. HPLC-FID area % precision ± 0.0-0.5%; SCF area % precision ± 1%.
[c] Supercritical fluid TAG analytical technique.[28]

Similar HPLC-FID factors were obtained for the LS of the palmitic, linoleic, and linolenic gravimetric series. Since for the Ln series FFA and TAG co-elute, gravimetric standards one without FFA and one without TAG, but with the other components (25 wt % each) were analyzed to obtain response factors for TAG and FFA. Average response factors for the LS of the palmitic, stearic, oleic, linoleic, and linolenic gravimetric series were: FAME, 1.14 ± 0.6; TAG, 0.86 ± 0.12; FFA, 0.91 ± 0.09; DAG, 0.97 ± 0.05; and MAG, 1.26 ± 0.04. These response factors showed that most of the needed correction for HPLC-FID area percent was required for TAG and MAG. The other LS, FAME, FFA, and DAG had correction factors close to unity.

We utilized the above results from HPLC-FID of gravimetric standards for analysis of experimental reaction mixtures resulting from lipid transformations. In Figure 2, a reaction mixture from glycerolysis of soybean oil for preparation of MAG[28] was resolved by the cyanopropyl HPLC column coupled with FID. This chromatogram is at the top of the figure. Reference chromatograms which include the starting soybean oil and other LS standards

reversed phase solid phase extraction before HPLC analysis. We have observed that free glycerol is retained by the cyanopropyl column. Utilizing the average response factors for LS presented above, the corrected HPLC-FID area percent is given for components of the glycerolysis mixture in Table 3. It is observed, that the corrected HPLC-FID area percent are in agreement with LS composition obtained by a supercritical fluid chromatography TAG analytical method.[28] Examination of the data obtained by application of the average response factors to HPLC-FID area percent should reflect that in the glycerolysis mixture the TAG and DAG also contained mixed FA species as opposed to only single FA species in the gravimetric standards. However, good agreement was still obtained between the LS composition from HPLC-FID and the supercritical analytical methods.

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# A SIMPLE HPLC ASSAY, WITH ULTRAVIOLET DETECTION, FOR DETERMINATION OF A MONOBACTAM ANTIBIOTIC

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## ABSTRACT

BMS-180680 is a monobactam antibiotic currently under development for the treatment of gram negative bacteria including Pseudomonas aeruginosa. Simple, rapid, sensitive, precise, and reproducible assay methods have been developed for the quantification of BMS-180680 in dog and rat plasma using aztreonam as the internal standard. The assay methods involve a single-step protein precipitation by addition of acetonitrile, followed immediately by centrifugation, after which the supernatant is evaporated to dryness (65°C) under a gentle stream of nitrogen. The residue is re-constituted in the mobile phase, transferred to a micro-WISP vial and injected on to a Zorbax $R_x$ C-18 HPLC column preceded by a guard column. Mobile phase comprised 17% acetonitrile and 83% of 40 mM dibasic ammonium phosphate and 6 mM tetrabutylammonium hydrogen sulfate. A small amount of tetrahydrofuran (20 mL/liter of mobile phase) was added and the apparent pH of the mobile phase was adjusted to 4.1 with 85% phosphoric acid. The column eluate was monitored by an ultraviolet

detector set at 252 nm. The nominal retention times were 8.0 and 9.0 min for aztreonam and BMS-180680, respectively. The lower limit of quantitation levels were 0.1 and 0.2 μg/mL in dog and rat plasma, respectively. The inter-assay and intra-assay precision values of the quality control (QC) samples were less than 6.4% relative standard deviation in both dog and rat plasma and the predicted concentrations of the QC samples deviated less than 10% of the corresponding nominal values. BMS-180680 was stable in dog and rat plasma at -20°C for at least 64 and 55 days, respectively and for at least three freeze/thaw cycles in either dog or rat plasma. Both BMS-180680 and aztreonam were stable in the autosampler for at least 59 hours at the room temperature. The assays were employed to measure BMS-180680 concentrations in heparinized dog and rat plasma samples obtained from a pharmacokinetic study.

## INTRODUCTION

Monobactam antibiotics such as aztreonam[1,2] and carumonam[3,4] have been demonstrated to show pharmacological activity against several gram negative organisms including Pseudomonas aeruginosa. BMS-180680, ((2R-[2α,3α{Z}])-3-[[[{-(2-amino-4-thiazolyl)-2-[(2-methyl-4-oxo-1-sulfo-3-azetidinyl)- amino]-2-oxo-ethylidene} amino]oxy]methyl]-6,7,dihydroxy-2-quinoxaline carboxylic acid), a monobactam antibiotic structurally similar to both aztreonam and carumonam, is currently being developed for the treatment of infections caused by gram negative organisms. In order to support toxicokinetic and pharmacokinetic studies, a simple high performance liquid chromatographic (HPLC) assay was developed and validated for the quantitation of BMS-180680 in dog and rat plasma using aztreonam as the internal standard.

## EXPERIMENTAL

### Materials

Analytical supplies of BMS-180680 and aztreonam, the internal standard (I.S.), were obtained from Bristol-Myers Squibb Co., New Brunswick, USA. Monobasic potassium phosphate, dibasic ammonium phosphate, 85% phosphoric acid, and concentrated hydrochloric acid (Fischer Scientific, Fairlawn, NJ, USA); acetonitrile and methanol (J.T. Baker Inc., Phillipsburg, NJ, USA); tetrahydrofuran (Burdick and Johnson Div., Muskegon, MI, USA); and tetrabutylammonium hydrogen sulfate (Aldrich Chemical Co., Milwaukee, WI, USA) were purchased

from the commercial suppliers. Control heparinized rat and dog plasma was obtained from Cocalico Biologicals Inc., Reamstown, PA, USA. Distilled water was deionized and filtered through a Millipore (Milford, MA, USA) Milli-Q system. All other solvents and chemicals used were of analytical grade and were used without further purification.

**Equipment**

The HPLC system comprised: a Waters Model 590 solvent delivery pump and a Waters 710B WISP autosampler (Water, Division of Millipore, Milford, MA, USA), and a model 785A Kratos programmable ultraviolet (UV) absorbance detector (Applied Biosystem, Inc., Ramsey, NJ, USA) set at 252 nm. The UV detector's output was interfaced through an analog-to-digital converter to a Model HP-3357 laboratory automation system (Hewlett Packard, Cupertino, CA, USA). Other equipment included a Model 720A digital pH meter (Orion, Cambridge, MA, USA), a Model IEC HN-SII centrifuge with a fixed-head 6/84 rotator (Damon/IEC Division, International Equipment, Needham Heights, MA, USA) and a turbo-vap LV evaporator (Zymark Co., Hopkinton, MA, USA).

**Standards and Quality Control Samples**

Stock concentrations of 400 μg/mL of BMS-180680 and 100 μg/mL of the I.S. were prepared in a buffer solution (pH 6) containing 100 mM potassium phosphate and 10 mM ammonium phosphate. The stock solution of BMS-180680 was used to prepare the standards in dog plasma (range, 0.1 to 40 μg/mL) and rat plasma (range, 0.2 to 40 μg/mL).

Three quality control (QC) samples were prepared separately in the dog (1.30, 11.3, and 30.8 μg/mL) and rat plasma (1.60, 16.2, and 30.3 μg/mL). These QC samples served to: (i) validate the assays for accuracy and precision, (ii) assess the plasma stability of BMS-180680 upon storage at -20°C and (iii) evaluate the freeze-thaw stability of BMS-180680.

**Preparation of Samples**

Aliquots (100 μL for the rat assay or 150 μL for the dog assay) of plasma samples (standards, QCs and blank samples) were transferred into disposable borosilicate culture tubes. Fifty μL (rat assay) or 30 μL (dog assay) of the I.S. solution (1 part of 100 μg/mL solution of aztreonam mixed with 1 part of 0.1 N

hydrochloric acid solution) was added and vortexed for a few seconds. Plasma proteins were precipitated by the addition of a 200 μL (rat assay) or 300 μL (dog assay) of acetonitrile. The samples were mixed thoroughly, followed by centrifugation at 2600 rpm for 10 minutes. Following centrifugation, the entire supernatant was transferred into another labelled tube and evaporated to dryness using a turbo-evaporator (65°C) under a gentle stream of nitrogen (7-10 psi). The residue was re-constituted in the mobile phase (130 μL) within 25-30 minutes. The entire mixture was transferred to a WISP vial with an insert. Approximately 75 μL of the mixture was injected on to the HPLC column.

**Mobile Phase**

The mobile phase comprised 17% acetonitrile and 83% of Milli-Q water containing 40 mM dibasic ammonium phosphate and 6 mM tetrabutylammonium hydrogen sulfate. In each liter of the mobile phase, a 20 mL volume of tetrahydrofuran was added. The apparent pH of the mobile phase was adjusted to 4.1-4.2 with 85% phosphoric acid.

**Chromatography**

The processed dog and rat plasma samples were analyzed using a 250 mm X 4.6 mm I.D. Zorbax $R_x$-C18 column with a particle size diameter of 5 μM (Mac-Mod Analytical Inc., Chadds Ford, PA, USA), immediately preceded by a 30 mm X 4.6 mm I.D. (ODS 5 μM particle diameter) pre-packed Brownlee™ guard column (Chrom Tech. Inc., Apple Valley, MN, USA). The mobile phase was delivered at a rate of 1.5 mL/min and the column eluate was monitored by an UV detector set at 252 nm.

**Data Processing**

The analog output of the UV detector was collected and processed by the HP-3357 laboratory automation system according to previously described procedures.[5] Briefly, the standard data (peak height ratio versus concentration) generated from each analytical run were weighted by the reciprocal of the corresponding concentrations and fit to a linear regression equation. The procedure described by Prescott was used to perform the outlier rejection of the standards.[6] The concentrations of unknown samples were determined by inverse prediction from the appropriate regression line.

**Validation Procedures**

The validation of the assay methods included documentation of assay linearity, accuracy, precision, lower limit of quantitation (LLQ), and specificity. The stability of BMS-180680 in the auto sampler, after repeated freeze/thaw cycles, and upon storage (-20°C) was also evaluated. All the validation procedures were performed separately for the dog and rat plasma matrices.

LLQ and specificity were evaluated concurrently using the plasma matrix from ten individual animals. For LLQ assessment, the ten individual samples were processed, both as blank samples and as samples spiked at LLQ, defined as 0.1 μg/mL for the dog and 0.2 μg/mL for the rat plasma matrix. Paired t tests were performed ($\alpha = 0.05$) to assess the statistical significance between peak heights for blank and those for spiked LLQ samples. For specificity evaluation, the individual paired chromatograms of blank and spiked samples were inspected to assess the degree of interference by endogenous constituents.

The accuracy and precision of the assays were assessed at concentrations in the upper, middle and lower quartile of the standard curve range, assaying at least six replicate samples for each concentration, on three different days. Accuracy was defined as the deviation of the mean observed concentration from the nominal and expressed as a percentage of the nominal concentration. Intra-assay precision estimate was expressed as the percentage relative standard deviation (RSD) of the predicted concentrations of the replicate samples from each group. The inter-assay precision estimate was calculated using the following equation: $100[(TrMS-EMS)/N]^{0.5}/GM$, where TrMS, EMS, and GM refer to treatment and error mean squares and grand mean, respectively.

The stability of BMS-180680 during storage at -20°C and after freeze-thaw cycles was evaluated using the plasma QC samples used to predict the accuracy and precision of the assay. The autosampler stability of BMS-180680 at the room temperature was evaluated by periodically injecting aliquots of the processed QC samples (dog: 2.5, 10 and 40 μg/mL; rat: 10, 20 and 40 μg/mL) over a 59 hr period and determining peak height as a function of time.

**Pharmacokinetic Application**

The assay methods were applied to analyze the unchanged BMS-180680 concentrations in plasma samples obtained from pharmacokinetic studies in dogs and rats.

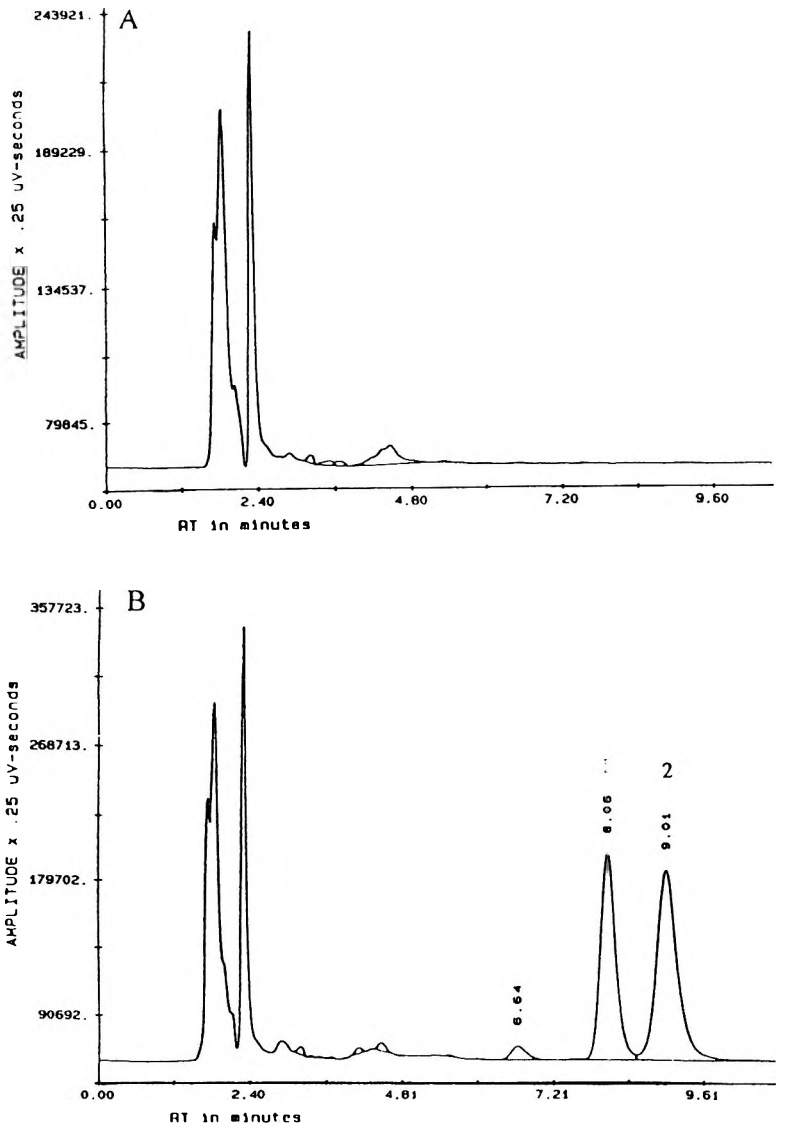


**Figure 1.** Representative chromatograms of a blank rat plasma [A] and a spiked standard containing aztreonam and BMS-180680 (20 μg/mL) [B] (aztreonam (1) and BMS-180680 (2) peaks correspond to retention times of 8 and 9 minutes, respectively).

**Table 1**

**Accuracy and Precision Data for the Analysis of BMS-180680 in Dog and Rat Plasma**

**(a) Intra-assay Accuracy and Precision**

| Species | Nominal Concentration (μg/mL) | Mean Predicted Conc'n (μg/mL) | N | Precision (% RSD) | Accuracy (%Deviation from nominal) |
|---|---|---|---|---|---|
| Dog | 30.8 | 31.3 | 9 | 6.4 | 1.7 |
| | 11.3 | 11.2 | 9 | 3.9 | -0.8 |
| | 1.30 | 1.18 | 9 | 6.1 | -9.1 |
| Rat | 30.3 | 33.3 | 9 | 3.5 | 9.9 |
| | 16.2 | 17.8 | 9 | 1.6 | 9.6 |
| | 1.60 | 1.57 | 9 | 3.8 | -2.0 |

**(b) Intra-assay Accuracy and Precision**

| Species | Nominal Concentration (μg/mL) | Mean Predicted Conc'n (μg/mL) | N | Precision (% RSD) | Accuracy (%Deviation from nominal) |
|---|---|---|---|---|---|
| Dog | 30.8 | 33.7 | 24 | 4.5 | -13.8 |
| | 11.3 | 11.7 | 24 | 3.7 | 3.3 |
| | 1.30 | 1.12 | 25 | 6.4 | 9.3 |
| Rat | 30.3 | 31.7 | 27 | 5.0 | 4.5 |
| | 16.2 | 17.2 | 27 | 5.5 | 6.0 |
| | 1.60 | 1.61 | 27 | 7.8 | 0.6 |

## RESULTS AND DISCUSSION

Since BMS-180680 is zwitter ionic in nature (presence of both acidic and basic functionalities in the molecule), a controlled pH environment of the mobile phase is essential to maintain satisfactory and consistent peak shape without peak splitting or peak tailing. In preliminary experiments, it was assessed that a mobile phase pH of about 4.1-4.2 was best suited for the separation of BMS-180680. At this pH value, the I.S. peak was also sharp and well separated from the BMS-180680 peak.

**Table 2**

**Stability of BMS-180680 inDog and Rat Plasma QC Samples on Storage at -20°C**

**(a) Dog Plasma**

| Time (Days) | Mean Predicted Concentrations (± DEV) at Various Concentrations | | |
|---|---|---|---|
| | **30.8 (μg/mL)** | **11.3 (μg/mL)** | **1.30 (μg/mL)** |
| 9 | 31.3 (1.7) | 11.2 (-0.8) | 1.18 (-9.1) |
| 44 | 26.5 (-14.1) | 11.6 (2.5) | 1.32 (2.6) |
| 64 | 25.9 (-15.9) | 9.38 (-16.8) | 1.23 (-4.5) |

**(b) Rat Plasma**

| Time (Days) | Mean Predicted Concentrations (± DEV) at Various Concentrations | | |
|---|---|---|---|
| | **30.3 (μg/mL)** | **16.2 (μg/mL)** | **1.60 (μg/mL)** |
| 1 | 29.8 (-1.6) | 16.0 (-1.5) | 1.48 (-2.0) |
| 22 | 34.6 (14.2) | 18.5 (14.2) | 1.71 (7.0) |
| 33 | 30.7 (1.5) | 17.9 (10.5) | 1.65 (3.1) |
| 55 | 29.1 (-4.1) | 16.3 (0.5) | 1.49 (-6.7) |

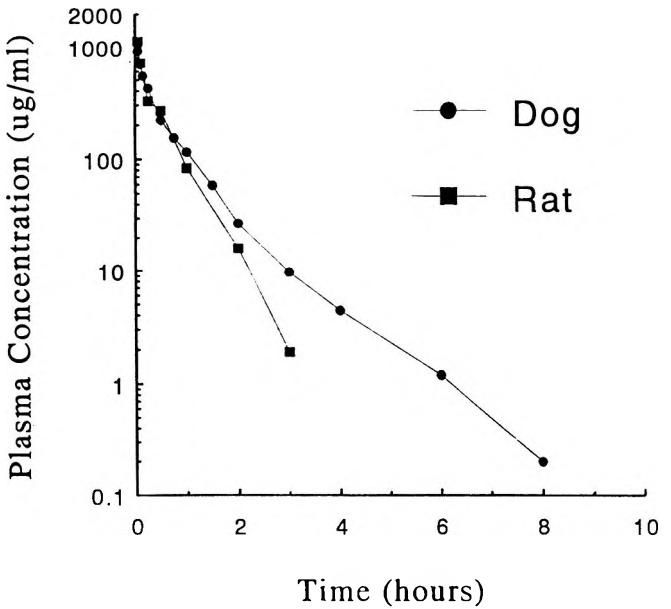


**Figure 2.** Plasma concentration-time profiles of BMS-180680 in a dog (closed circles) and in a rat (closed squares) receiving intravenous doses of 100 and 200 mg/kg of BMS-180680, respectively.

Representative chromatograms of a blank and a spiked standard in the rat plasma matrix are shown in Figure 1. Inspection of the chromatograms obtained from rat plasma indicated no interference at the peaks of interest by endogenous materials. Similar inferences were drawn from the chromatograms obtained from dog plasma.

The nominal retention times for the peaks of I.S. and BMS-180680 were 8 and 9.0 min, respectively. The standard curves demonstrated linearity ($R^2 > 0.995$) over the concentration range of 0.1 to 40 μg/mL in the dog plasma and 0.2 to 40 μg/mL in rat plasma.

LLQ was established as the lowest standard curve concentration, 0.10 μg/mL for the dog and 0.20 μg/mL for the rat plasma assay. The mean value for LLQ in the dog plasma assay had a precision of 14.5% RSD and an accuracy of 1.2% deviation. The mean value for LLQ in the rat plasma assay had a precision of 8.9% RSD and an accuracy of 5% deviation.

The intra-assay (within-day) and inter-assay (between-day) accuracy and precision data for the dog and rat plasma assays are shown in Table 1. The inter- and intra-assay (between days) precision estimates (RSD) for the dog plasma assay were less than 7% and for the rat plasma assay were less than 8%. The inter- and intra-assay accuracy estimates were within ± 14% for either of the two assays.

The autosampler stability of BMS-180680 in the processed dog or rat plasma samples was demonstrated over a period of 59 hr. The freeze/thaw stability study indicated that BMS-180680 in dog or rat plasma was stable (within ± 15% of nominal values) through three freeze/thaw cycles. Finally, BMS-180680 was found to be stable in the dog and rat plasma at -20°C for at least 2 months (Table 2).

The developed and validated assays in dog and rat plasma were utilized in the analysis of plasma samples from pharmacokinetic studies. A representative plasma concentration-time profile of intact BMS-180680 following a single intravenous dose in dogs (100 mg/kg) and rats (200 mg/kg) are shown in Figure 2.

## CONCLUSIONS

The assay methods developed for the quantification of BMS-180680 in the dog and rat plasma are simple, specific, sensitive, accurate, precise, and reproducible. The advantages of this assay includes the rapidity of the sample analysis due to a minimal sample preparation and the use of small sample volumes.

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# DETERMINATION OF CHLOROQUINE AND DESETHYLCHLOROQUINE IN BIOLOGICAL SAMPLES USING PERFUSION CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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## ABSTRACT

A high-speed reversed phase HPLC method for the determination of chloroquine and desethylchloroquine in biological samples is presented. Perfusion chromatography, based on the use of flow-through particles made of cross-linked polystyrene/divinylbenzene, allowed to increase the flow-rate of the mobile phase to 4 mL/min, resulting in base-line separation of *bis*-desethylchloroquine, desethylchloroquine, chloroquine, and the internal standard in less than 3 minutes. As the column material withstands prolonged exposure to high pH conditions, the pH of the mobile phase could be adjusted to 9.5, enabling direct determination of chloroquine and desethylchloroquine by fluorescence detection after elution; therefore, no post-column alkalinization to obtain the pH of optimal fluorescence was required. Interday as well as intraday reproducibility for chloroquine concentrations ranging from 31.3 to 500 ng/mL in plasma was lower than 7%. The applicability of the method is illustrated by measuring blood concentrations of chloroquine and desethylchloroquine in blood during 1 week after oral intake of 500 mg chloroquine.

## INTRODUCTION

Since its introduction forty years ago, chloroquine has been the most important drug in the treatment and prophylaxis of malaria. Although the emergence of chloroquine-resistant strains of *Plasmodium falciparum* and *Plasmodium vivax* has reduced its efficiency, chloroquine remains an important antimalarial drug in tropical Africa and Asia.[1,2] Chloroquine is also being used in the treatment of rheumatoid arthritis and systemic lupus erythematosus.[3,4] The determination of chloroquine concentrations in biological samples is important for several reasons, such as the assessment of patient compliance, the determination of pharmacokinetic data and the prevention of toxic blood concentrations after prolonged use, especially in the case of the treatment of rheumatoid arthritis. Reliable analysis methods are also required for quality control of chloroquine preparations. Spectrophotometry,[3] fluorimetry,[6] GLC,[7-9] HPLC,[10-18] and ELISA[19] have all been used for the determination of chloroquine in biological samples. Recently, a CE method has been described for the bioanalysis of antimalarial drugs.[20] In HPLC, fluorescence detection offers superior sensitivity to UV detection, but requires normal phase chromatography[16,17] or reversed phase chromatography combined with post-column alkalinization.[18] Packing consisting of cross-linked polystyrene/divinylbenzene withstands prolonged exposure to high pH conditions, so that pH can be adjusted directly to the pH of optimal fluorescence, making post-column alkalinization redundant. In addition, the presence of flow-through pores of 6000-8000 Å in the polymeric packing of perfusion chromatography columns allows the mobile phase to flow through the packing material, while diffusion is possible in smaller pores (800-1500 Å) that are connected to them.[21] The presence of these pores allows to increase the flow-rate of the mobile phase, resulting in high sample turn-over. The high-speed HPLC analysis described in this paper is an improvement of a method developed earlier in our laboratory.[18]

## MATERIALS AND METHODS

### Reagents

Chloroquine diphosphate and diethylamine were obtained from Sigma (St. Louis, USA); desethylchloroquine and 6,8-dichloro-4-(1-methyl-4-diethylaminobutylamino)quinoline were kindly supplied by Sterling Drug Inc. (Rensselaer, N.Y.), and *bis*-desethylchloroquine by Rhône-Poulenc; acetonitrile and tetrahydro-furan were obtained from BDH (Poole, England)

and Sigma-Aldrich (Gillingham, England), respectively. Dichloromethane was from Rathburn (Hojbjerg, Denmark) and di-sodium tetraborate from UCB (Leuven, Belgium). All reagents were used as received.

**Chromatographic Conditions**

The HPLC system consisted of a Waters (Millford, Mass.) 600E System Controller, a Rheodyne injector equipped with a 100 μL loop, and a fluorescence detector (Model SFM 25, Kontron; Ex. 331 nm, Em. 381 nm). Chromatograms were recorded and processed using the Maxima 820 computer program. The column used was a 4.6-mm diameter x 100 mm length (1.7 mL column bed volume) POROS ® R/H (PerSeptive Biosystems, Cambridge, MA). The packing consisted of 10 μm flow-through particles of cross-linked polystyrene/divinylbenzene. Mobile phase A consisted of an aqueous buffer containing 20 mM di-sodium tertraborate, 0.1 % diethylamine and 20% acetonitrile (pH adjusted to 9.5 with 2N NaOH). Mobile phase B consisted of 100% tetrahydrofuran. Separation was obtained using the following gradient: after an initial stage of 0.3 min in mobile phase A, mobile phase B was increased to 4% and, at 0.6 min, to 10% by a step gradient; from 0.6 to 3 min, mobile phase B was increased by a linear gradient from 10 to 15%; to flush the column, mobile phase B was further increased over 0.5 min to 40 %, followed by an isocratic stage of 1 min and a return to initial conditions over 0.5 min; column re-equilibration in mobile phase A was obtained in 1 min. The flow rate of the mobile phase amounted to 4 mL/min. Both mobile phases were filtered and degassed continuously by He sparging. The injection volume amounted to 100 μL. The column was kept at a temperature of 60 °C.

**Sample Preparation**

Samples were prepared according to the method described earlier.[18] In short, plasma (150 μL) or blood (75 μL) samples were alkalinized with 0.5 mL NaOH 2N after addition of 100 μL internal standard solution (6,8-dichloro-4-(1-methyl-4-diethylaminobutyl-amino)quinoline, 1 μg/mL in HCl 0.2 N). After extraction with 7.0 mL dichloromethane and centrifugation (4000 rpm, 10 min), the water layer was discarded and the organic layer evaporated to dryness under a gentle stream of air. The residue was dissolved in an aliquot of mobile phase A and 100 μL was injected into the HPLC system. To prevent adsorption of chloroquine on glass, 0.1% diethylamine was added to the NaOH solution used for alkalinization.[22]

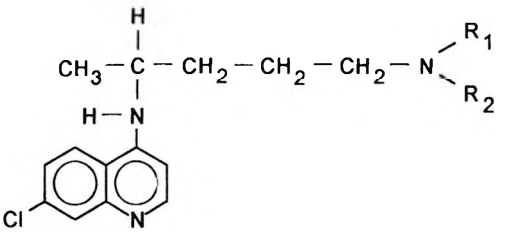

**Figure 1**. Structure of Chloroquine ($R_1$ = $R_2$ = $C_2H_5$) and its main metabolites desethylchloroquine ($R_1$ = $C_2H_5$; $R_2$ = H) and *bis*-desethylchloroquine ($R_1$= $R_2$ = H).

### Calibration Plot

Stock solutions of desethylchloroquine, chloroquine and the internal standard were prepared at a concentration of 1 mg/mL in MeOH/$H_2O$ (50/50). Drug-free plasma was spiked with aliquots of the stock solutions of desethylchloroquine and chloroquine, and handled similarly as the samples. A calibration graph was constructed in which concentration was plotted against the peak/area-ratio. All concentrations calculated are the average of two separate determinations.

## RESULTS AND DISCUSSION

For the development of an HPLC bioanalysis of chloroquine and its main metabolite (Figure 1), fluorescence detection is preferred to UV detection because of superior sensitivity. Fluorescence in aqueous solution shows a maximum at a light alkaline pH,[6] which is illustrated in Figure 2. While chloroquine and desethylchloroquine show an obvious maximum between pH 8.5 and 10, the pH dependence of the fluorescence of the internal standard was smoother. A pH of 9.5 was chosen for all further experiments. Unfortunately, conventional reversed phase columns only withstand mobile phases with a maximum pH of 8.0, thus requiring post-column alkalinization for optimal fluorescence. To enable direct determination of chloroquine and its metabolites, packing material of cross-linked polystyrene/divinylbenzene was used. This material withstands prolonged exposure to extreme pH conditions, so that the pH of the mobile phase could be directly adjusted to the pH of optimal detection (pH 9.5). The through-pores present in the packing material allowed to increase the speed of the mobile phase to 4 mL/min, without increasing the back-pressure, offering the additional advantage of rapid analysis.

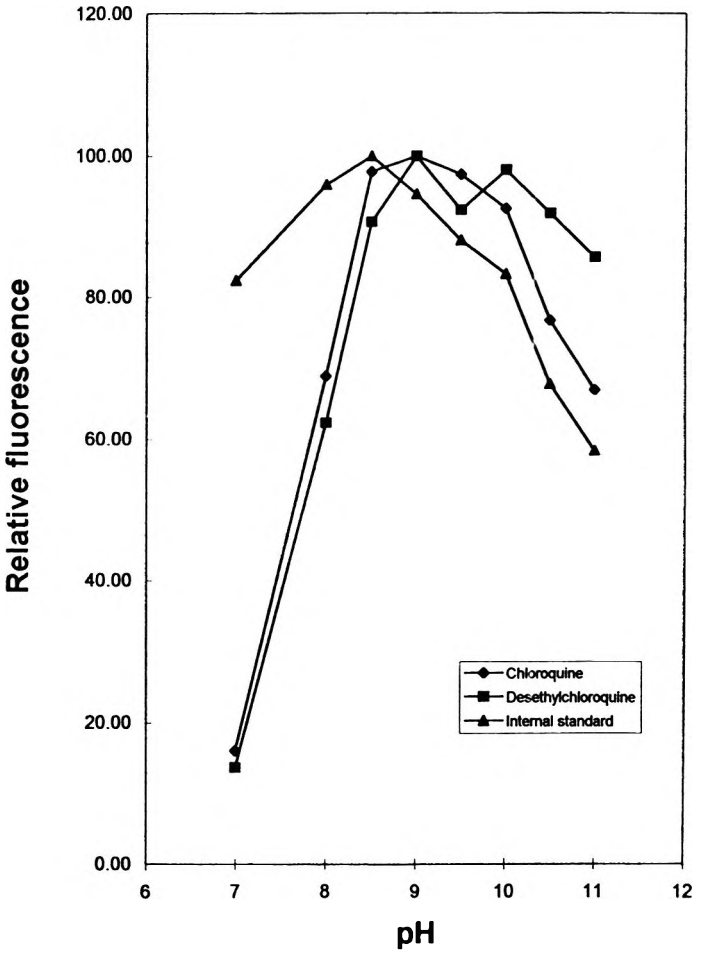


**Figure 2**. Relative fluorescence of chloroquine, desethylchloroquine and the internal standard as a function of pH (max. = 100%).

Using a gradient, base-line separation of chloroquine, desethyl-chloroquine, bisdesethylchloroquine and the internal standard could be obtained in about 2.5 min; re-equilibration in mobile phase A took 1 min, resulting in a total analysis time of less than 4 min. A wash-step up to 40% of mobile phase B could be included, as described in the materials and methods section. This short analysis time allowed to increase the sample turn-over and to decrease the apparatus occupation time. Typical chromatograms obtained by the final method are shown in Figure 3.

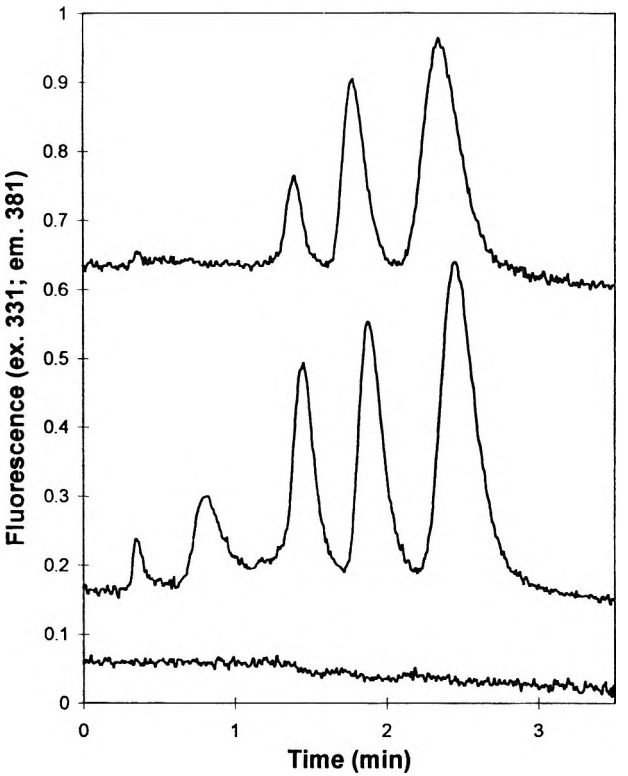


**Figure 3**. Chromatograms of a blank plasma sample (lower panel), a test solution containing *bis*-desethylchloroquine (rt = 0.8 min), desethylchloroquine (rt = 1.45 min), chloroquine (rt = 1.9 min), and the internal standard (rt = 2.45 min), and a sample prepared from plasma spiked with 31.3 ng/mL desethylchloroquine and 125 ng/mL chloroquine (upper panel).

The precision of the assay was assessed for chloroquine (31.3 - 500 ng/mL) and desethylchloroquine (15.6 -125 ng/mL) spiked in pooled human plasma, and is expressed as relative standard deviation in Table 1 (chloroquine) and Table 2 (desethylchloroquine). Calibration plots of chloroquine and desethylchloroquine showed good correlation ($r > 0.998$). From the chromatograms, a detection limit, which corresponded to a peak height of 3 times the noise of the baseline, was calculated as approximately 5 ng/mL for chloroquine and desethylchloroquine. As only 150 μL plasma or 75 μL blood was used in this study, increasing the sample volume will definitely improve the sensitivity of the assay.

**Table 1**

**Reproducibility of Chloroquine Bioassay**

| Concentration Added (ng/mL) | Concentration Found (ng/mL) | RSD* (%) |
|---|---|---|
| **Intraday Variation (n = 7)** | | |
| 500 | 510 | 3.8 |
| 250 | 245 | 5.4 |
| 125 | 126 | 4.5 |
| 62.5 | 58.3 | 3.4 |
| 31.3 | 33.9 | 3.7 |
| **Interday Variation (n = 5)** | | |
| 500 | 498 | 3.2 |
| 250 | 240 | 4.7 |
| 125 | 120 | 3.0 |
| 62.5 | 60.3 | 2.2 |
| 31.3 | 31.1 | 4.5 |

RSD = Relative Standard Deviation.

**Table 2**

**Reproducibility of Desethylchloroquine Bioassay**

| Concentration Added (ng/mL) | Concentration Found (ng/mL) | RSD* (%) |
|---|---|---|
| **Intraday Variation (n = 7)** | | |
| 125 | 129 | 7.3 |
| 62.5 | 62.9 | 9.8 |
| 31.3 | 32.7 | 5.5 |
| 15.6 | 15.4 | 5.9 |
| **Interday Variation (n = 5)** | | |
| 125 | 129 | 4.3 |
| 62.5 | 62.8 | 14 |
| 31.3 | 31.1 | 3.6 |
| 15.6 | 15.5 | 11 |

RSD = Relative Standard Deviation.

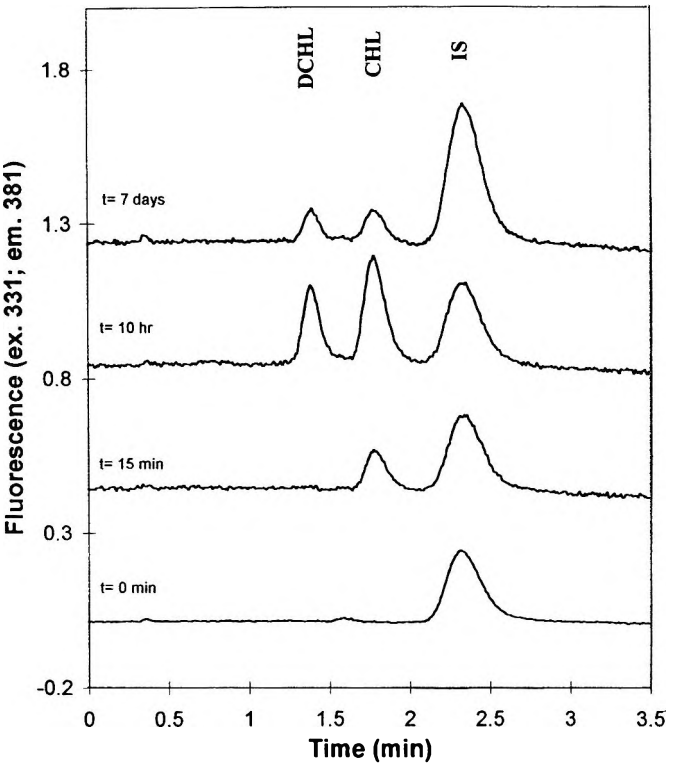


**Figure 4**. Chromatograms displaying the elimination and metabolism of chloroquine as a function of time after oral intake of 500 mg chloroquine at t=0 (DCHL = desethylchloroquine, CHL = chloroquine, IS = internal standard).

The applicability of the method is demonstrated by analyzing blood samples from a human subject, following an oral dose of 500 mg chloroquine. Figure 4 shows representative chromatograms at different time points; it can be seen that there is no substantial difference between chromatograms obtained from a blood sample and the chromatograms obtained from spiked plasma samples (Figure 3). As the extraction recovery from plasma and blood samples was reported to be in the same range,[18] total blood concentrations were calculated from a calibration plot made up in plasma. The use of total blood samples (75 μL) instead of plasma samples offers the advantage that concentrations are 2-5 times higher than those in plasma, due to accumulation in erythrocytes.[23] The concentration-time profile shown in Figure 5 illustrates that the detection limit mentioned is sufficient to calculate basic pharmacokinetic parameters.

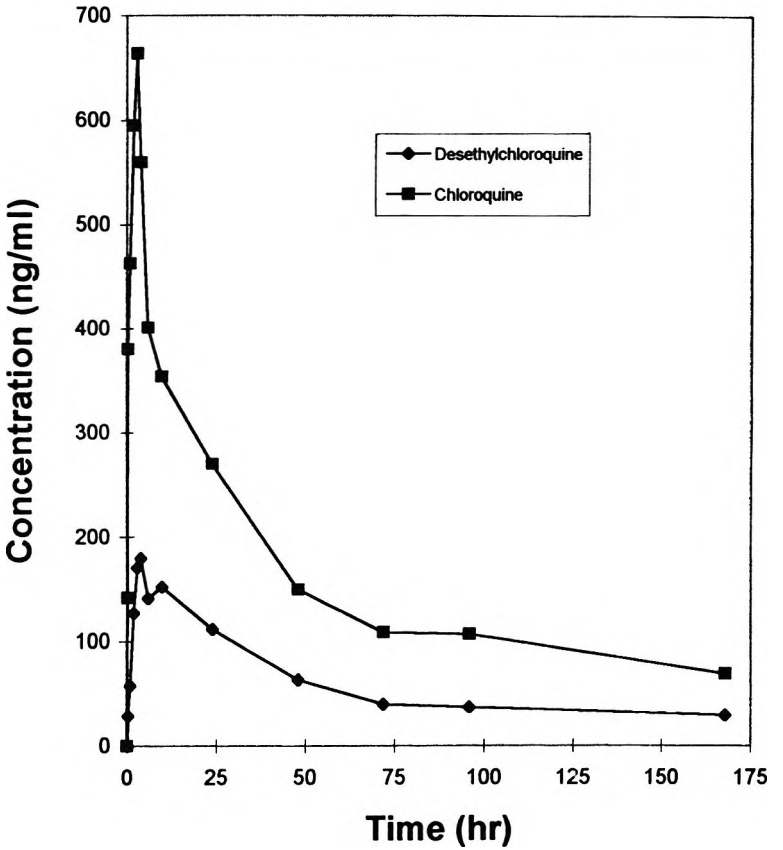


**Figure 5**. Concentration-time profile of chloroquine and desethylchloroquine during 1 week after oral intake of 500 mg chloroquine.

In summary, these results demonstrate that chloroquine and desethylchloroquine can be determined in biological samples by a high speed HPLC method based on the use of perfusion chromatography; the high pH possible on the cross-linked polystyrene/divinylbenzene packing opens the possibility to adjust the pH of the aqueous mobile phase to that of optimal fluorescence of chloroquine, enabling direct determination after elution from the column. The method can easily be adapted for quality control of chloroquine preparations.

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# DETERMINATION OF DIFLUBENZURON AND TEFLUBENZURON IN FISH FEED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A simple method for the determination of diflubenzuron and teflubenzuron in fish feed by HPLC is presented. The samples were extracted with acetone - tetrahydrofurane and diluted with tetrahydrofurane, prior to the HPLC. The lower limit of quantification was 0.25 and 0.4 g/kg. for diflubenzuron and teflubenzuron in fish feed, respectively.

## INTRODUCTION

Diflubenzuron (DFB) and teflubenzuron (TFB) are insecticides belonging to a group of compounds that act by inhibiting chitin biosynthesis and deposition.[1] DFB and TFB are effective in the control of a variety of insects.[2] TFB is applied at regular intervals throughout the growing season of some fruits to control insect pests. It can also be used to reduce the incidence of "contaminants" such as mites on harvested fruit.[3,4]

Infestation with sea lice, *Lepeophtheirus salmonis* and *Caligus elongatus*, is a growing problem in fish farming. The lice damage the skin, causing unthriftiness, and in severe cases the lice may even result in death of the fish. The parasite may also transmit microbial pathogens, and infestations may impact upon wild salmonids. A comparison of infestations between wild and farmed fish shows an usually higher prevalence and abundance on the latter, indicating enhanced transmission of sea lice under farm conditions.[5,6,7]

A range of methods (chemical, physical and biological) have been introduced for controlling sea lice. Treatment with, chemotherapeutics has included the use of dichlorvos, trichlorfon, azamethiphos, carbaryl, ivermectin, pyrethrum and hydrogenperoxide.[6]

The pesticides DFB from Solvay Duphar and TFB from Cyanamid which can be given orally (in feed) to salmon, are two of the newest drugs introduced in the treatment of sea lice in salmon. Methods for the determination of DFB in fish tissues,[8] in environmental samples,[9] foresty substrates,[10] and residues in cabbage under sub-tropical field conditions,[11] have been described. Only one method for the determination of TFB in fish tissues has been described.[8] However, none of the published methods appear to be applicable to medicated fish feed.

The purpose of the present study was thus to develop a simple HPLC method for the routine analysis of DFB and TFB in fish feed.

## MATERIALS AND METHODS

### Materials and Reagents

Samples of commercial fish feed free of DFB and TFB were used. For testing of the method, medicated (commercial) fish feeds containing DFB and TFB were used.

All chemicals and solvents were of analytical or HPLC grade. The fish feeds were produced by Ewos and Skretting. Diflubenzuron (Solvay Duphar), was donated by Ewos Aqua A.S. (Skårer, Norway) and teflubenzuron (Cyanamid) was donated by Skretting (Stavanger, Norway). Stock solutions (1mg/mL) of DFB and TFB were prepared by dissolving the compounds in tetrahydrofurane. The solutions were stored in the refrigerator.

**Chromatographic Conditions**

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system (with acetonitrile as flushing liquid) equipped with a Lauda RMT6 cooler (12°C) from Messgeräte Werk Lauda (Lauda Königshafen, Germany), and a LC 235C diode array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 250 nm (fixed wavelength). The integration was carried out using the software programme Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon).

The analytical column (stainless steel, 15 cm x 4.6 mm. ID) and guard column (stainless steel , 2 cm x 4.6 mm. ID), were packed with 5 μm particles of Supelcosil LC - ABZ (Supelco, Bellefonte, PA, USA). The guard column was connected to an A.318 precolumn filter with an A-102X frits (Upchurch Scientific, USA).

The mobile phase was a mixture of acetonitrile-water-tetrahydrofurane (50 : 37 : 13). The flow rate was 1 mL/min.

**Sample Pretreatment**

Exactly 0.5 g ground feed was weighed into a 50 mL graduated centrifuge tube with screw cap (Nunc, Roskilde, Denmark), and made up to 50 mL volume with acetone-tetrahydrofurane (6 : 4). The sample was blended, placed in an ultrasonic bath for 5 min (at room temperature) and then left in the extraction fluid for 10 min before again being placed in an ultrasonic bath for 5 min. The sample was mixed and centrifuged for 3 min (3000 rpm). A 1 mL volume of tetrahydrofurane was added to 100 μl of the supernatant and the mixture then blended. The samples (10 μl) were injected into the HPLC system at intervals of 12 min for the determination of DFB and TFB.

**Calibration Curves And Recovery Studies**

The calibration curves for DFB and TFB were obtained by spiking feed samples with standard solutions, to yield 0.3, 0.5, 0.75, 1.0, and 2.0 g/kg and 0.5, 0.75, 1.0, 2.0 and 3.0 g/kg, of DFB and TFB in fish feed, respectively. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked samples with those of standard solution. The linearity of the standard curves for DFB and TFB in fish feed was tested using peak-height measurements.

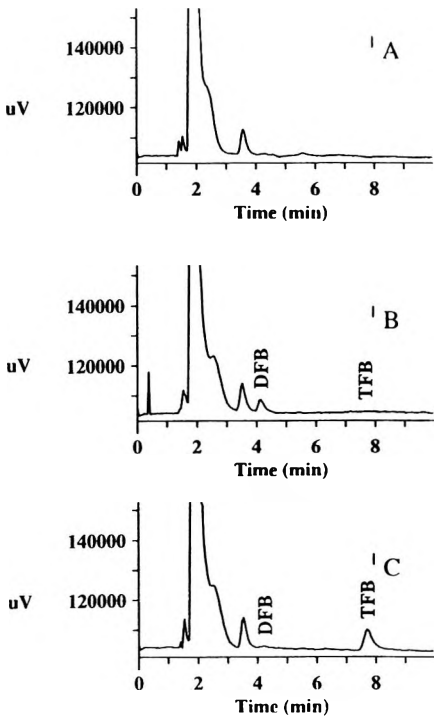


**Figure 1**. Chromatograms of extracts from 0.5g fish feed. A) drug-free fish feed, B) "real" sample of fish feed contains 0.6g/Kg DFB, C) "real" sample of fish feed contains 2.2 g/Kg TFB.

## RESULTS AND DISCUSSION

Chromatograms of commercial samples of clean feed and of medicated fish feed containing DFB and TFB are shown in Figures 1. The standard curves were linear in the investigated areas; 0.3 to 2.0 g/kg for DFB and 0.5 to 3.0 g/kg for TFB.

The linearity of the standard curves for DFB and TFB in fish feed was 0.999 and 0.997, respectively, when using the external standard method of calculation. The precision and recovery for DFB and TFB from fish feed were also calculated, and are shown in Table 1.

Table 1

| Material | No. of Samples | Amount in Spiked Samples (g/kg) | Recovery % DFB Mean | DFB S.D. | TFB Mean | TFB S.D. |
|---|---|---|---|---|---|---|
| Feed | 8 | 0.30 | 91.43 | 1.84 | | |
| (1g) | 8 | 0.75 | 92.97 | 2.44 | | |
| | 8 | 0.75 | | | 90.79 | 0.86 |
| | 8 | 3.00 | | | 90.27 | 1.61 |

S.D. = standard deviation.

The extraction procedures were validated, and showed good recovery of DFB and TFB. The recovery varied from 91.4 to 93 % and from 90.8 to 90.3% for DFB and TFB , respectively. The precision of these recovery studies varied from 1.8 to 2.4 % and from 0.9 to 1.6 % for DFB and TFB in fish feed, respectively.

For DFB, the limit of quantification was 0.25 g/kg and the limit of detection 0.15 g/kg. The limit of quantification for TFB was 0.4 g/kg and the limit of detection 0.3 g/kg.

In Norway, commercial medicated fish feed usually contains about 0.6 and 2 g/kg of DFB and TFB, respectively. The potential number of samples that can be dealt with per day is limited only by the duration of the HPLC procedure. The assay shows good precision when using the external standard method.

The method described is rapid, simple, robust and sufficiently sensitive,with good recovery. The quantification is linear over a wide concentration range.

## ACKNOWLEDGEMENTS

We are grateful to the Norwegian Research Council for financial support.

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# USE OF BENZOYL HYDRAZINE REAGENT FOR MONOSACCHARIDE DETERMINATION BY HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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## ABSTRACT

Benzoyl hydrazine derivatives of reducing carbohydrates were analyzed by high performance capillary electrophoresis with on-tube ultraviolet detection. The derivatives of 9 reducing monosaccharides were completely separated in *ca.* 30 min. using a capillary tube (50 μm i.d., 62 cm) and 200 mM borate buffer, pH 10.8, as carrier. On-column UV detection at 220 nm was able to achieve sensitive detection down to 30~40 femtomole levels. The experimental parameters for optimum precolumn are discussed along with derivatization and separation. It was demonstrated that this method allows quantification of reducing carbohydrates with high accuracy and reproducibility.

## INTRODUCTION

The carbohydrate moieties of glycoproteins or glycolipids perform a number of functions in a wide variety organisms, including protection from proteolysis, receptors for bacteria and viruses, and other functions related to immunological specificity and cellular differentiation.[1] Compositional analysis of the complex carbohydrates is of fundamental importance in structural studies of these compounds. A variety of modern chromatographic techniques have been applied to this task, including gas chromatography,[2] supercritical fluid chromatography,[3] and liquid chromatography.[4] More recently, high performance capillary electrophoresis (HPCE) has shown promise as a new analytical method for carbohydrate analysis because of rapid and quite high-resolution.[5,6] In many cases, HPCE has demonstrated separation far superior to that of other chromatographic methods. Carbohydrates are usually neutral and do not possess chromophores; they, therefore, pose problems for CE separation and optical detection methods. One common strategy to realize separation and detectablility is to perform derivatization to introduce charged groups and chromophores. Various precolumn derivatizations have already been used in capillary zone electrophoresis (CZE),[5,7-12] for example, reducing monosaccharide were derivatized to N-(2-pyridyl)glycamines which formed charged borate complexes with the borate in the buffer solution.[5] Another method for the analysis of carbohydrate with CE is based on fluorogenic reagents, such as 3-(4-carboxybenzoyl)2-quinolinecarboxaldehyde or 3-benzoyl-2-naphthaldehyde.[6] They are based on the reaction of the primary amine group of the derivatization reagents with the carbonyl group of the sugar or the primary amine group of the sugars with the carbonyl group of the derivatization reagents. Hydrazine reagents have been used as carbonyl group reagents for the determination of sugars because of rapid, specific, and high derivative yield.[13,14]

In this paper, benzoyl hydrazine was adapted as a derivatization reagent for monosaccharide analysis. The derivatization, separation and quantitation of sugars were investigated.

## MATERIALS AND METHODS

Benzoyl hydrazine was purchased from Serva Co. (Germany) and used without further purification. All carbohydrate samples were of the highest grade commercially available from Packard-Becker. B. V., Netherlands. Ethanol and acetic acid, used as reaction medium for precolumn derivatization, were reagent grade from the Beijing Chemical Factory (Beijing, China).

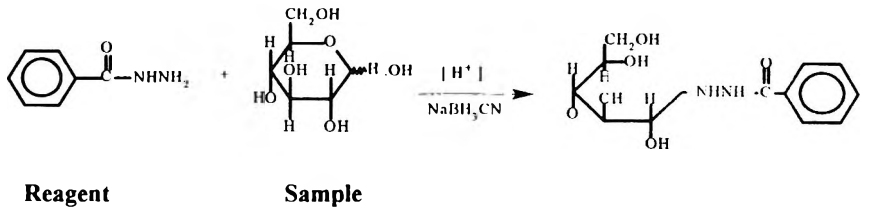


**Figure 1**. Reaction of benzoyl hydrazine with carbohydrate.

Sodium cyanoborohydride was purchased from Fluka (Switzerland) and calf serum fetuin from Sigma. Redistilled, deionized water was used to prepare the buffer. Borate solutions were prepared by dissolving pellets of boric acid in redistilled water and adjusting the pH to the indicated value with NaOH. Saccharide standards were dissolved in 75% (v/v) ethanol to give 5 mM solutions; they were stored at 4 °C.

## Apparatus

HPCE was carried out with the Spectra PHORESIS 1000™ system with PC1000 vision 3.0 software from Thermo Separation Products, Inc., Fremont, CA. Their breadboard included the scanning focus 1000 detector, a 0 to 30 kv DC power supply, an air thermostatic capillary compartment, and a vacuum sample injector. Fused silica capillary of 50 μm i.d. and 375 μm o.d. was purchased from Yongnian Photoconductive Fiber Factory (Hebei, China). All experiments were carried out with uncoated fused silica capillaries. A new capillary was first flushed with 1 M sodium hydroxide, followed by water and then the running buffer. The running buffer was renewed after 5-6 runs, and the capillary column was flushed with fresh buffer before each injection in order to ensure reproducible separations.

## Derivatization Procedure with Benzoyl Hydrazine (see Fig. 1)

All procedures were performed in a 0.5 mL polypropylene tube with a screw cap. To 20 μL of an ethanol solution, containing 100 nmol of reducing sugars, was added 90 μL of ethanol containing 1~2 % acetic acid and 1% sodium cyanoborohybride followed, with mixing, by 10 μL of a specific concentration of benzoyl hydrazine in 75% ethanol, such that the range of molar ratios of saccharide to benzoyl hydrazine was between 1:2.5 to 1:20 for

sugar standard and 1:200 for hydrolysis sample of glycoproteins. The mixture was heated at 60 °C for 300 min. in a water bath and then cooled to room temperature. The derivatized sugars were directly injected into the capillary for separation.

### Hydrolysis of Glycoprotein

According to the method described previously,[14] acid hydrolysis of calf serum fetuin was carried out as follows. Samples were dissolved with 200 μL of water in screw-cap Teflon tubes and 200 μL of 8 M TFA was added. The samples were hydrolyzed in a boiling-water bath for 6 h. The tubes were cooled, and the samples were dried by nitrogen, dissolved in ethanol, and subjected to derivatization as described above.

## RESULTS AND DISCUSSION

### Optimization of Derivatization Conditions

The reaction of hydrazine with aldehyde groups is a well known, specific acid-catalyzed nucleophilic addition,[15] which yields hydrazone products. When the reaction is conducted in the presence of a reducing agent, sodium cyanoborohydride ($NaBH_3CN$), the hydrazone amine was alkylated as shown in Figure 1. To achieve optimum detection sensitivity and reproducibility, it was necessary to optimize pre-column derivatization conditions with respect to acid concentration, reaction time and the molar ratios of the hydrazine to sugar. The three most common monosaccharides (mannose, glucose and galactose) were examined for determination of the optimum conditions. Figure 2 illustrates the relationship between the peak areas of the three sugar derivatives and the concentration of acetic acid over the range 0.1-10 % (v/v). It shows that the peak areas increased markedly at acid concentrations greater than 2.5%. However, the mannose derivative showed a decreased peak area at high acid concentration (>5%, v/v). In addition, much higher concentration of acid resulted in alteration of carrier pH, which destroyed the peak reproducibility. Therefore, the acetic concentration was fixed, usually, at 1~2% (v/v).

To determine the proper ratio of benzoyl hydrazine reagent to sugar, the molar ratio of benzoyl hydrazine to sugar was varied from 2.5:1 to 20:1 at 3h, 60 °C. As demonstrated in Figure 3, onsets of curvatures were observed.

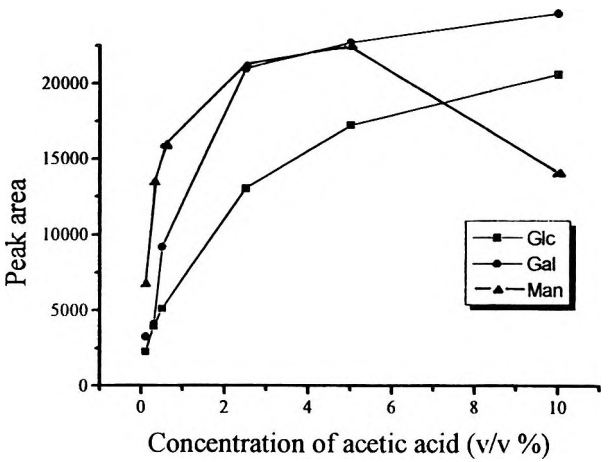


**Figure 2**. Effect of acetic acid concentration on the peak areas of precolumn sugar derivatives. Derivatization performed at 50 °C for 3 h. Reaction products electrophoretic conditions: applied voltage, 16 kv; capillary, 44 cm total length, 36 cm to the detector, 50 μm i.d.; buffer, 150 mM borate, pH10.5; ambient temperature, 30 °C.

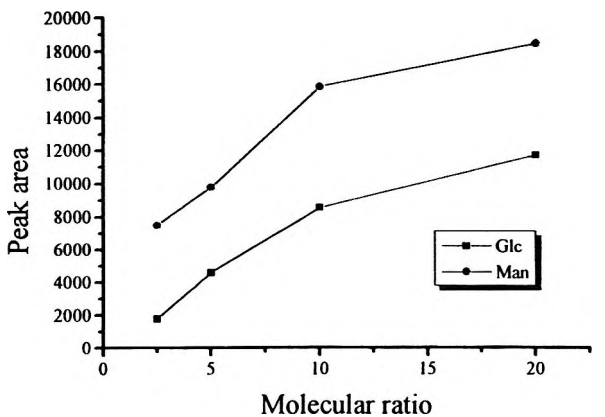


**Figure 3**. Effect of molecular ratio of saccharide to benzoyl hydrazine on derivatization reaction at 60 °C for 3 h. Electrophoretic conditions as in Figure 2.

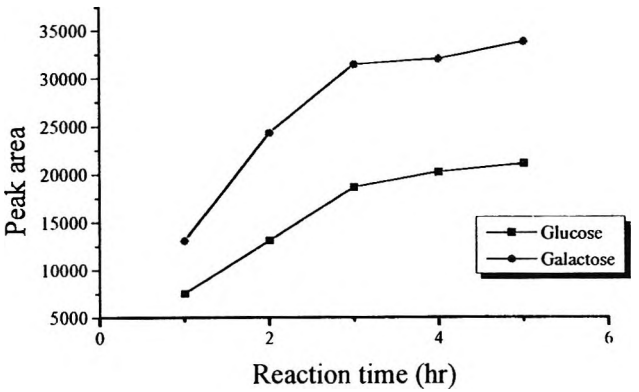


**Figure 4**. Effect of reaction time on precolumn derivatization reaction at 60 °C, Electrophoretic conditions as in figure 2.

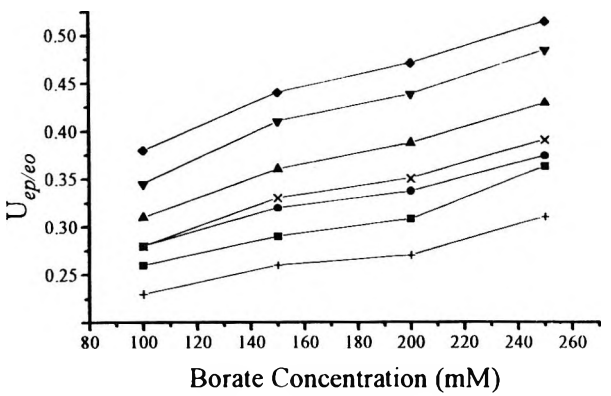


**Figure 5**. Plots of relative mobility *vs.* concentration of borate buffer at pH10.2. Conditions: fused silica capillary, 70 cm × 50 μm i.d.; voltage, 18 kV; detection wavelength, 220 nm; ambient temperature, 25°C. ■ Rhamnose, ● Xylose, Δ Glucose, ∇ Fucose, ◇Galactose, + N-acetylgalactosamine, × Ribose.

Although the benzoyl hydrazine was eluted at the electroosmotic flow without interfering with the sugar derivative separation, a large amount of excess reagent resulted in current waving, which decreases the experimental reproducibility. Thus, the optimum molecular ratio was usually twenty-fold.

The optimum temperature for hydrazone formation was found to be 60 °C; at lower temperatures, the reaction was slow while, at higher temperature, it was very difficult to maintain a constant acid concentration. The reaction time was varied to study its effects on reaction efficiency at 60 °C, acetic concentration 1.5 % (v/v). Here again, convex curves were observed (Figure 4). This result is different from that with Dns-hydrazine, used for labeling sugars, where the derivatization recovery decreased with extended the reaction times after reaching the maximum yield.[16] In the interest of time economy, three hours was adopted. At this reaction time, the relative peak area was about 91% of that at 5 h for all these carbohydrate derivatives.

## Optimization of the Electrophoretic Analysis Condition

### Precision of relative mobility

Separation in CZE is achieved via the distinct migration velocities of analytes under the influence of an electric field. An analyte is typically identified by its migration time ($t_R$) in the electropherogram or electrophoretic mobility ($\mu_{ep}$). They are both dependent upon electrophoretic conditions with respect to applied voltage, capillary length and column temperature. Therefore, the migration time precision and reproducibility are usually poor.[17] In our experiment, a relative mobility parameter ($\mu_{ep/eo}$) was adopted to describe the analyte migration, as defined below:

$$\mu_{ep/eo} = \frac{\mu_{ep}}{\mu_{eo}} \tag{1}$$

where $\mu_{ep}$ is the electrophoretic mobility of the analyte, $\mu_{eo}$ is electroosmotic mobility of the carrying solution. When contributions from the relaxation effect are neglected, they both can be expressed by following equation (17):

$$\mu_{ep} = \frac{2\varepsilon}{3\eta}\zeta_a f(\kappa a) \tag{2}$$

$$\mu_{eo} = \frac{\varepsilon}{\eta}\zeta_c \tag{3}$$

where $\varepsilon$ is the permitivity of carrying solution, $\eta$ the viscosity of the carrying solution, $\zeta_a$ the potential of the analyte, $k$ the thickness of diffusion layer, a is

the "radius" of the analyte, f($\kappa a$) is a function dependent upon the shape and $\kappa a$ of the analyte in the buffer and $\zeta_c$ is the Zetta potential between the capillary and carrying solution.

Combination of eqn. (1), (2), and (3) leads to an expression for $\mu_{ep/eo}$:

$$\mu_{ep/eo} = \frac{2\zeta_a}{3\zeta_c} f(\kappa a) \tag{4}$$

From equation (4), it can be seen that the relative mobility is dependent upon $\zeta_a$ in a giving buffer, but not $\varepsilon$ and $\eta$. Therefore, the variation in temperature and the accidental error was decreased. In our study, the electrophoretic mobility of the analyte and electroosmotic mobility of the carrying solution can be calculated by following equations, respectively.

$$\mu_{ep} = \frac{lL}{V}\left(\frac{1}{t_R} - \frac{1}{t_0}\right) \tag{5}$$

$$\mu_{eo} = \frac{lL}{V}\frac{1}{t_0} \tag{6}$$

where $l$ is the distance between the inlet of the capillary tube and the detector, $L$ the total length of the capillary tube, $t_0$ the retention time of the neutral marker, $t_R$ the migration time of the analyte and V is the applied voltage.

Thus, the relative mobility of the analyte can be determined by combining equations (5) and (6) to give:

$$\mu_{ep/eo} = \frac{t_0 - t_R}{t_R} \tag{7}$$

From equation (7), it can be seen that the relative mobility of analyte is independent of the length of capillary and the applied voltage, which the variation in the set of experimental conditions was decreased. Therefore, quantitative precision and good reproducibility can be obtained. A reproducibility comparison of $t_R$, $\mu_{ep}$ and $\mu_{ep/eo}$ obtained in the same capillary is shown in Table 1.

**Table 1**

**Comparison of Precision of $t_R$, $\mu_{ep}$ and $\mu_{ep/eo}$**

| Sugars | ----- R.S.D. % (n = 6) ----- $t_R$ | $\mu_{ep}$ | $\mu_{ep/eo}$ |
|---|---|---|---|
| Rhamnose | 0.98 | 0.68 | 0.17 |
| Xylose | 1.0 | 0.68 | 0.23 |
| Glucose | 0.96 | 0.84 | 0.28 |
| Fucose | 1.1 | 0.63 | 0.30 |
| Galactose | 1.1 | 0.70 | 0.22 |

Conditions: applied voltage, 16.0 kv; capillary length, 36 cm to the detector; i.d., 50 μm; buffer, 200mM borate, pH 10.8; ambient temperature, 30°C.

As shown in Table 1, the precision of each of the parameters, as indicated by RSD's is impressive. However, the precision of $\mu_{ep/eo}$ is clearly superior to that of $t_R$ and $\mu_{ep}$.. An explanation for this might be the thermal effect (Joule heat) in the experimental conditions. Both $t_R$ and $\mu_{ep}$ are rather temperature-sensitive, but not $\mu_{ep/eo}$., In the following experiments, the relative mobility was used to specify the analytes.

**Retention behavior**

Since monosaccharides are usually neutral, CZE is not directly applicable. Using borate as the buffer, they can be converted to anionic borate complexes by complexation of hydroxyl groups with borate ion.[5] Solute migration in this investigation is dependent on a number of factors, the most important of which is probably the anionic borate complex formation. To achieve optimum separation, it was necessary to optimize complexation conditions with respect to borate concentration, pH and column temperature.

**Effect of concentration of the buffer solution**

The dependence of relative mobility of sugar derivatives on borate concentration was examined at pH 10.2 and shown in Figure 5. It is observed that increasing the borate concentration resulted in an increase of the relative

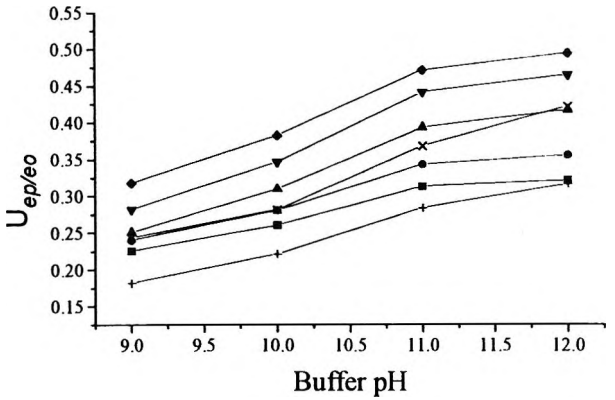


**Figure 6**. Dependence of the derivative relative mobility on pH with 150mM borate as carrying electrolyte. Conditions: applied voltage, 18 kv; capillary, 70cm total length, 62cm to the detector, 50 μm i.d., ambient temperature, 30°C. ■ Rhamnose, ● Xylose, ▲ Glucose, ▼ Fucose, ◆ Galactose, + N-acetylgalactosamine, × Ribose.

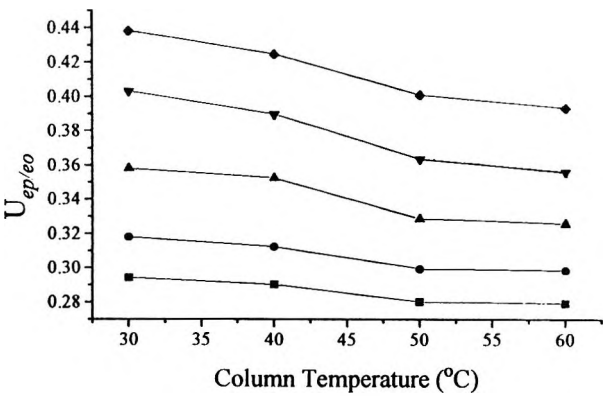


**Figure 7**. Effect of column terperature on the derivative relative mobility. Conditions: applied voltage, 18 kv; capillary, 70cm total length, 62cm to the detector, 50 μm i.d.., buffer, 150mM borate, pH 10.2; ambient temperature, 30°C. ■ Rhamnose, ● Xylose, ▲ Glucose, ▼ Fucose, ◆ Galactose.

mobility. Increasing the concentration of carrying electrolyte decreases the thickness of the diffusion layer, consequently decreasing the Zetta potential ($\zeta_c$), thus increasing the relative electrophoretic mobility according to Equation 4.

The plot shows that high borate concentration provides optimum selectivity by magnifying small steric differences between closely related isomers. However, satisfactory separation of sugars could not be achieved by varying the borate concentration alone, since baseline noise became significant above 250mM.[5]

**Effect of pH of the buffer solution**

Formation of a borate complex is facilitated with alkali. Figure 6 shows the effects of buffer pH on the relative mobilities of sugars. The general trend observed is that as the pH increased, the relative mobility increased. This can be attributed to the increase of potential of the analyte ($\zeta_a$). Higher pH values benefit formation of borate-sugar complexes, and result in the analyte potential increase.

**Effect of operating temperature on the separation**

The effects of varying column temperature on the separation of the five sugars is depicted in Figure 7. It was observed that, in general, as the separation column temperature increased, the relative migration mobility of sugars decreased. This can be attributed to an increase in the Zeta potential. When temperature increased, the diffusion coefficient D increased and, hence, the Zeta potential increased.

The plot shows that slightly lower temperature provides the optimum selectivity. This result is contrary to the result that high temperature gives better selectivity with underivatized monosaccharides.[18]

Figure 8 depicts the electropherogram of the reaction mixture of 9 reducing monosaccharides, obtained under optimized conditions. Separation was completed in about 30 min. and large, high resolution, numbers of theoretical plates were readily achieved for monosaccharides. The values ranged from 100,000 to 400,000 theoretical plates per meter.

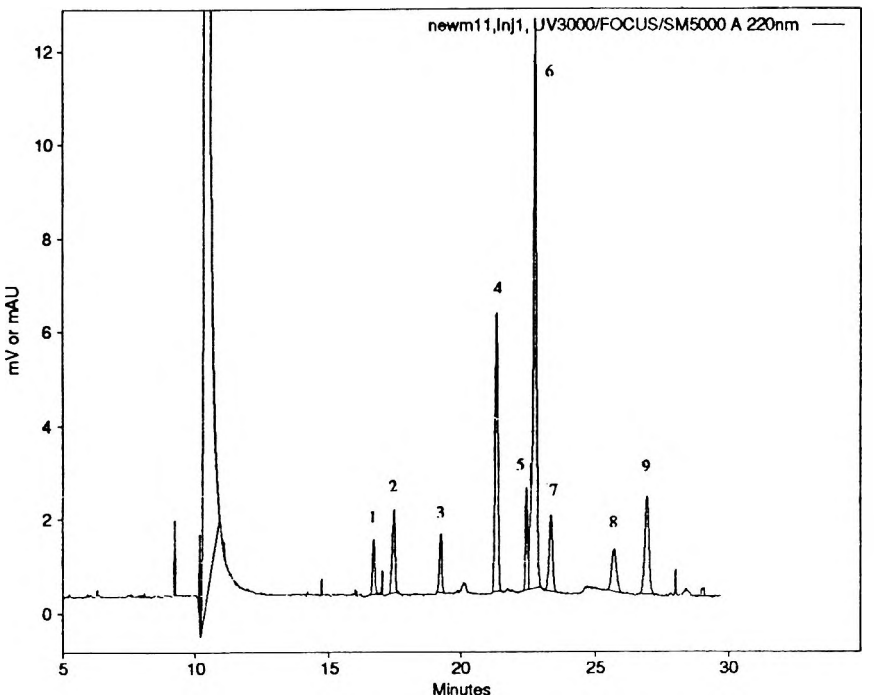


**Figure 8.** Electroopherogram of 9 standard reducing monosaccharide derivatives. Conditions: injection, vaccum 8 sec.; temperature, 30 °C; capillary length 70 cm (62 cm to detector); i.d. 50 μm; applied voltage, 20 kv; buffer, 200 mM borate, pH10.8; detection, 220 nm. 1- N-acetylgalactosamine, 2- Rhamnose, 3-Lyxose, 4-Xylose, 5-Manose, 6-Glucose, 7-Arabose, 8-Fucose, 9-Galactose.

## Reproducibility of Quantitative Analysis

### Sensitivity and linearity of response

The sugar derivatives possess intense ultraviolet absorption. The optimum wavelength is 220 nm. The detection limits of sugars were determined with a signal-to-noise ratio of 3, as shown in Table 2. The low detection limit is attributed to the high selectivity and low nanoliter sample injection volumes. The linearity of the detector response was investigated by injection of progressively diluted samples of sugar derivatives.

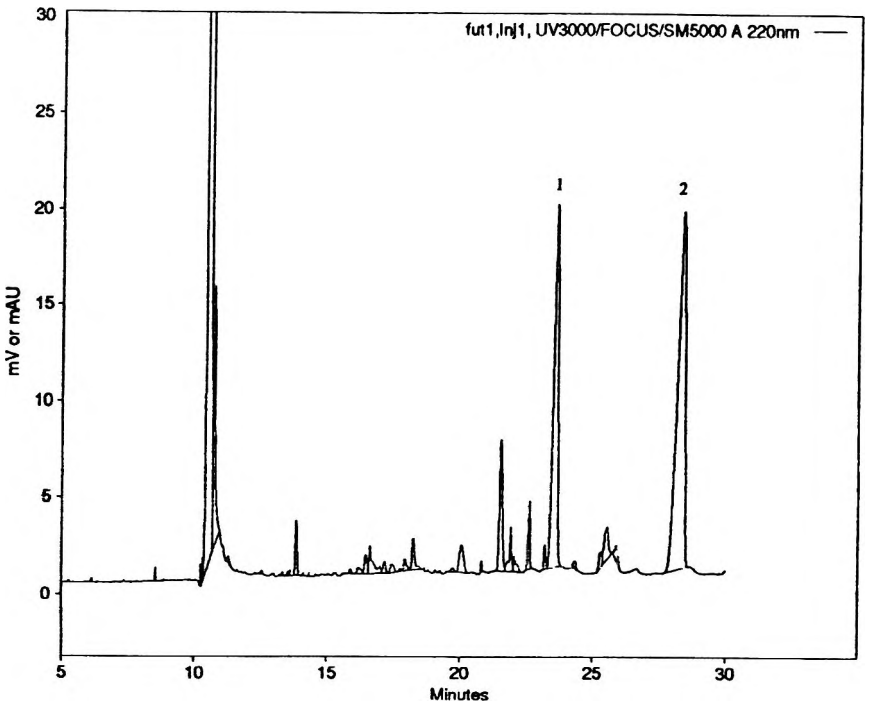


**Figure 9**. Analysis of the constituent monosaccharides of calf fetuin by CZE. The analysis condition as in Fig 8. 1 - Manose, 2 - Galactose.

Using β-indoleacetic acid as the internal standard. the response for the standard monosaccharides concentration to the relative peak area was linear over the range 10-700 ppm with satisfactory correlation coeffiecy $R^2$ =0.998 and determination was reproducible.

## Analysis of the Monosaccharide Components of Glycoproteins

The well characterized glycoprotein fetuin was hydrolyzed and the released sugars were derivatized with benzoyl-hydrazine to test the accuracy and the sensitivity of the analytical method. Figure 9 shows the electropherogram for a hydrolysate of fetuin in 4M THF.

The value obtained for the molar proportion, liberated by heating for 6 h. in 4M trifluoroacetic acid at 100 °C, was mannose:galactose, 0.64:1, which is in excellent agreement with the previously reported values (18,19), demonstrating the reliability of this method.

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# BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

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Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097, USA. Tel.: (954) 973-8516; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(7), 1141-1147 (1997)

# LIQUID CHROMATOGRAPHY CALENDAR

**1997**

**APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California.** Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

**APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes**, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

**MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California.** Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

**MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California.** Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

**MAY 27 - 28: IInd Miniaturisation in Liquid Chromatography versus Capillary Electrophoresis Conference, University of Ghent, Ghent, Belgium.** Contact: Prof. Dr. W. Baeyens, Faculty of Pharmaceutical Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

**MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan.** Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994.

**MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY.** Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

**MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois.** Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

**JUNE 1 - 4: 1997 International Symposium, Exhibit & Workshops on Preparative Chromatography: Ion Exchange, Adsorption/Desorption Processes and Related Separation Techniques, Washington, DC.** Contact: J. Cunningham, Barr Enterprises, 10120 Kelly Road, Box 279, Walkersville, MD 21793, USA. (301) 898-3772; FAX: (301) 898-5596.

**JUNE 16 - 19: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach. L'Electrophorese Capillaire, Methode de Routine pour le Contrôle Qualité des Medicaments: Approche Pratique, Monpellier, France.** (Training course given in two languages) Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Fac. de Pharmacie, F-34060 Montpellier Cedex 2, France. Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ.montp1.fr.

**JUNE 20: Enantiomeric Separation in Capillary Electrophoresis, a short course given by Dr. K. Altria, Glaxo-Wellcome, Ware, UK, in Montpellier, France**. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Fac. de Pharmacie, F-34060 Montpellier Cedex 2, France. Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ.montp1.fr.

**JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York.** Contact: T. Noce, Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

**JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas.** Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

**AUGUST 2 - 9: 39th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency Denver, Denver, Colorado**. Contact: Patricia Sulik, Rocky Mountain Instrumental Labs, 456 S. Link Lane, Ft. Colleins, CO, USA. (303) 530-1169; Email: gebrown@usgs.gov.

**SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada**. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (703) 231-8222.

**SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio.** Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

**OCTOBER 6 - 10: Validation d'une Procedure d'Analyse, Qulification de l'Appareillage; Application a la Chromatographie Liquide, Montpellier, France.** (Training course given in French) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr

**OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia.** Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

**OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California.** Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

**OCTOBER 26 - 29: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Boubletree Hotel, Washington, DC**. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

**OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain.** Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

**OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri.** Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

**NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico.** Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

**NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey.** Contact: S. Good. EAS. P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

**1998**

**MARCH 1 - 6: PittCon '98, New Orleans, Louisiana.** Contact: PittCon '98, Suite 332. 300 Penn Center Blvd., Pittsburgh, PA 15235-5503. USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

**MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas.** Contact: ACS Meetings. 1155 16th Street, NW. Washington, DC 20036-4899. USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri**. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279. Walkersville. MD 21793. USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

**JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington.** Contact: K. Grant. Math/Science Div. Columbia Basin College. 2600 N 20th Ave, Pasco, WA 99301. USA. Email: reglmtgs@acs.org.

**JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia.** Contact: D. J. Abraham, Virginia Commonwealth Univ. Dept of Med Chem. P. O. Box 581. Richmond. VA 23298. USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

**AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts.** Contact: ACS Meetings. 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

**SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland.** Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY. UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

**SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania.** Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

**NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Res. Triangle Pk, North Carolina.** Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204. Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

**1999**

**MARCH 7 - 12: PittCon '99, Orlando, Florida.** Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

**MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif**. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

**AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana**. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee.** Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville. TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

**2000**

**MARCH 5 - 10: PittCon 2000, Chicago, Illinois.** Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

**MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

**JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Seattle, Washington**. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596.

**AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

**2001**

**APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

**AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois**. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**2002**

**APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida**. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

**SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

**2003**

**MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**2004**

**MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

**AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

## 2005

**MARCH 13 - 17: 229th ACS National Meeting, San Diego, California.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

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1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

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1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

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