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# Determination of antibiotics and antimicrobial agents in human serum by direct injection onto silica liquid chromatographic columns

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**Abstract**: Antibiotics and antimicrobial agents are therapeutically monitored by the direct injection of untreated human serum onto silica columns. This simple approach does not lead to rapid column deterioration since the eluents utilized are almost totally aqueous and, consequently, prevent precipitation of the serum protein on the column. Column capacity of the drugs studied varies due to the mutual interaction of the eluent ionic buffer and organic modifiers. The influence of the number of serum injections on column-back pressure, column capacity and quantitative analyses is discussed.

**Keywords**: *High-performance liquid chromatography; silica gel with reversed-phase eluents; direct injection of serum; antibiotics.*

## Introduction

Determination of antibiotics in body fluids usually involves a number of steps which might require the use of solvent extraction, evaporation, solid extraction, ultrafiltration or column switching. These sample step operations contribute significantly to the total analysis time and work. Three recent references review the sample preparative strategies for drugs in biological fluids [1–3].

There is an obvious advantage in the reduction of tedious sample pre-treatment steps. Direct application of aqueous samples (urine or serum) onto silica columns is such an approach. However, the major concern with this approach is the precipitation of proteins in aqueous mobile phases containing organic modifiers (methanol or acetonitrile) at concentrations greater than 15%. A secondary concern is that the drug of interest must usually be present in serum at concentrations above 1 μg/ml and have sufficient molar absorptivity so that it can then be easily detected by a UV detector without further sample concentration. These requirements are generally met by the commonly administered antibiotics, with the general exception of the aminoglycosides. Some of the other detection methods used in therapeutic drug monitoring and on-line trace enrichment techniques have been reviewed [2].

There are several literature examples in which untreated serum samples have been directly injected onto chromatographic columns for purposes of monitoring drug levels.

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The earliest example is the use of an ion-exchange column for the detection of flucytosine an antifungal agent and furosemide a diuretic agent [4]. Furthermore, salicylic acid and naproxen were monitored in serum by direct injection onto reversed-phase and silica chromatographic columns [5]. In addition, there are several papers in which a drug present in serum at sufficiently high concentrations, can be injected in a relatively small volume of serum, i.e. 1 μl, without apparent deterioration of the chromatographic column [6–8]. The applicability of a narrow-bore liquid chromatographic system for the trace enrichment and direct plasma injection of the anti-cancer drug etoposide has been demonstrated [9]. A recently developed liquid chromatographic reversed-phase packing with a small-particulate internal surface was described for use in direct serum injections [10]. The apparent advantage of the smaller internal surface is that it prevents proteins from accumulating on the chromatographic support.

Unfortunately, the cited examples are very specific and consequently, do not give a generalized approach to direct serum injection.

This article highlights the author's general approach of direct serum injection onto silica chromatographic columns for the analysis of a wide structural variety of antibiotics and antimicrobial agents important in therapeutic monitoring.

## Experimental

*High-performance liquid chromatography*

The HPLC system consisted of a model 110A pump, an in-line filter, a saturator column filled with 37-μm silica (all from Beckman Instruments, Altex Scientific Operations, Berkeley, California) and a Waters Resolve® 10 cm × 8 mm i.d. column packed with 5-μm silica (Waters Associates, Milford, Massachusetts). The flow rate was 1–3 ml/min.

All commercially available silica columns should be thoroughly washed with isopropanol prior to equilibration with aqueous eluents. Detection was by means of a Model 773 UV detector (Kratos Analytical Instruments, Ramsey, New Jersey). Sample injections of 20 μl were made using a model 420B autoinjector (Perkin–Elmer Corp., Norwalk, Connecticut). Data were processed with the aid of an H-P 3357 laboratory computer (Hewlett–Packard Co., Avondale, Pennsylvania).

*Materials*

All of the antibiotics used were USP reference standards except for vancomycin which was obtained from Eli Lilly Co. (Indianapolis, IN). Human serum used in the spiking studies was purchased from Flow Laboratories (McLean, Virginia).

## Results and Discussion

Nearly all methods developed for the analysis of antibiotics in biological fluids are based on the use of reversed-phase chromatographic columns [3]. Only under special circumstances has it been possible to develop reversed-phase methods based totally on aqueous eluents [5]. Otherwise, eluents with an organic modifier especially greater than *ca* 15% over numerous injections will cause precipitation of the serum protein, followed by obstruction and rapid deterioration of the chromatographic column. Consequently, the methodological approach in the analysis of antibiotics has been to deplete the sample of protein prior to chromatography. Besides the increased analysis time in using this approach, quantitative errors due to losses of drugs in protein have been noted [11, 12].

The success of developing chromatographic methods for bulk and formulated drugs using silica with aqueous eluents has been documented [13–15]. Silica chromatographic systems using less than 15% organic modifier have been developed for the 16 antibiotics studied (Fig. 1) which are representative of penicillins, cephalosporins, aminoglycosides, polypeptides, glycopeptides, polyene and sulfonamide antibiotics. The capacity factors of these agents with a mobile phase composition of 0.1% phosphoric acid are summarized (Table 1). When monitoring (210–300 nm) a serum blank that has been injected directly, serum components interfere up to a capacity factor of *ca* 3.0 (Fig. 2). Nearly all the compounds, except for metronidazole, are clearly resolved from the serum background. However, metronidazole, as well as nystatin and amphotericin B, can be monitored at >320 nm with little or no serum background. In order to optimize the chromatographic system for any particular antibiotic, i.e. shorten or lengthen the chromatographic run time or resolve metabolites, four eluent variables should be taken into account. The chromatographic variables that affect capacity factors and selectivity of silica are buffer composition, solute pK, the pH and composition of the eluent. For instance, in order to decrease the capacity factors of the two cephalosporins, cephradine and cephalexin, an organic modifier, such as acetonitrile can be added to the eluent (Table 1 and Fig. 3).

Another useful technique in decreasing the retention time of a component is to increase the ionic strength of the eluent. A variation of this approach which can also affect the selectivity is to use long-chain quaternary ammonium ions such as acetyltrimethylammonium bromide [16]. An increase in eluent pH, with all other chromatographic parameters being constant, decreases the capacity factor for acids, whereas, it increases the capacity factors for basic compounds [13]. For the antibiotics studied, the relationship with capacity factor, solute pK and pH is more complex since the majority that were studied are amphoteric (Table 1). Nevertheless, the dominant separation mechanism seems to be due to the ion-exchange capacity of silica [14]. Additional substance–eluent-interactions of silica in polar eluents also have been demonstrated [17].

In all the experiments, human serum was directly injected or, in some instances in which the serum was not clear, it was diluted 1:1 with eluent prior to injection. After *ca* 50 injections of 20 μl each, the chromatographic column becomes saturated with proteins and fats, consequently, these components are no longer adsorbed by the chromatographic column and elute in the void volume (Fig. 3). The author's experience has been that protein saturation of the column has no apparent detrimental effect on the quantifying of solutes. Comparison of solute peak heights and areas of spiked serum samples to that of external standards showed that all measurements were within experimental error and similar to findings reported in the literature [5]. Generally, after 200 injections, eluents containing low concentrations of either acetonitrile or methanol will denature the serum protein, decreasing the column porosity which leads to increased column back-pressure. If the replacement of the column frits does not decrease the column back-pressure it is recommended that the column should be replaced.

In the specific instance where a metabolite elutes under the protein peak or where peak splitting occurs, a precolumn containing silica that is vented or periodically changed should be used [18]. Precolumn venting is a column switching method that when properly used diverts serum protein from the analytical chromatographic column. Peak splitting has been observed when analysing serum for various drugs and appears to be directly related to the amount of serum injected onto the chromatographic column [5]. Peak

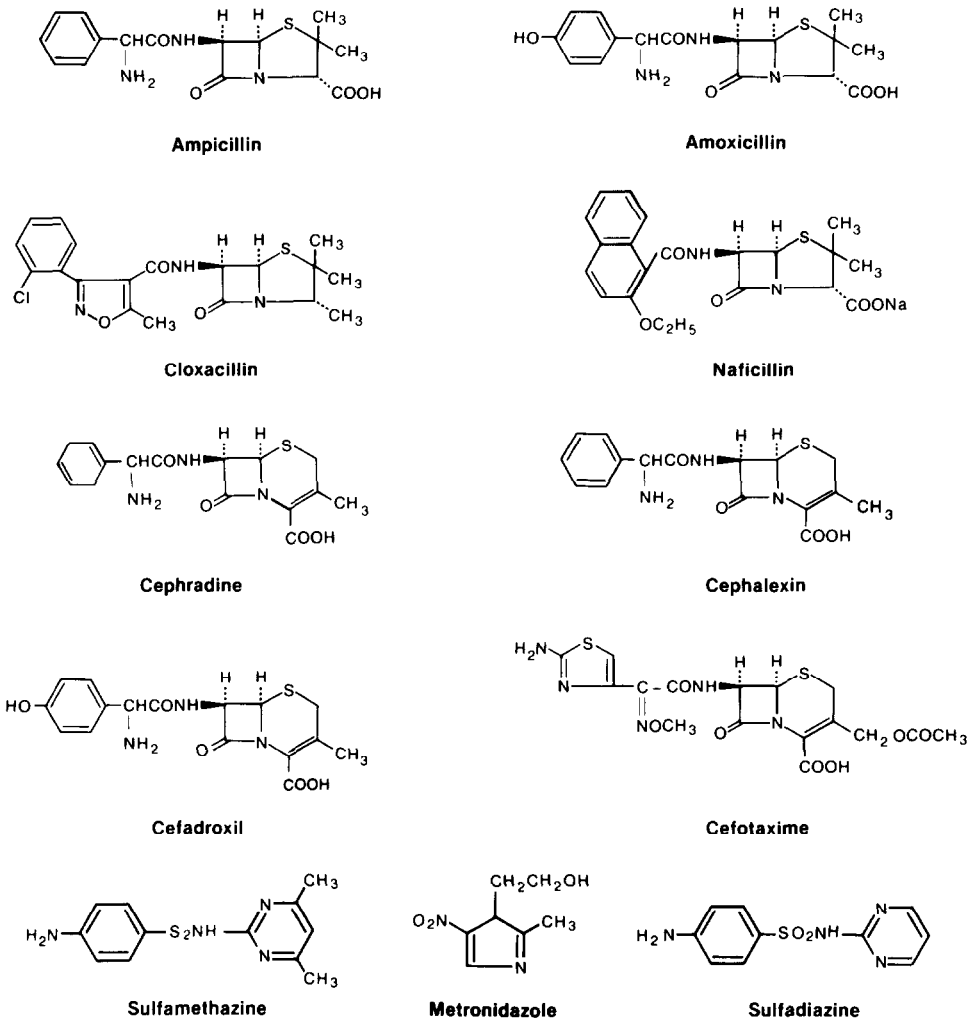
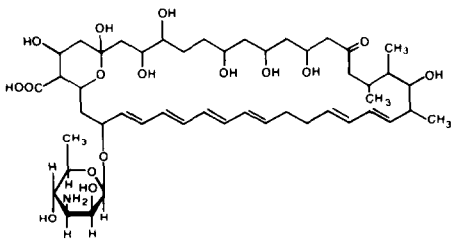


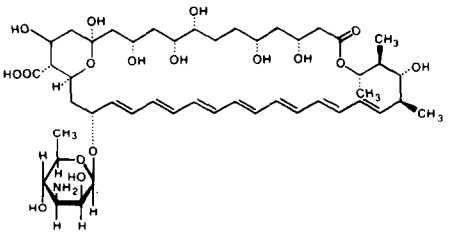

**Figure 1**
Structure of the antibiotics studied.

splitting can be minimized by injection 20 μl or less of serum. This may be a useful observation especially in developing stereoselective assays of chiral drugs since human serum albumin binding has been shown to be stereoselective [19].

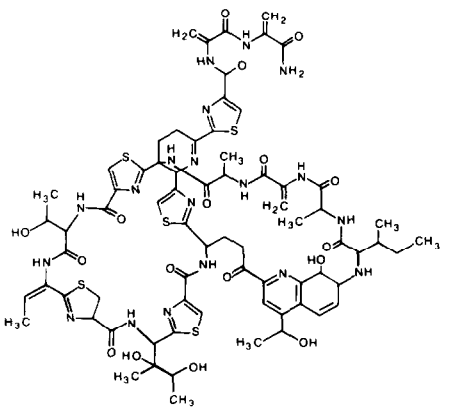
Blank human serum spiked at 20 and 100 μg/ml of the individual antibiotics were analysed with the chromatographic eluent of acetonitrile–0.1% v/v phosphoric acid (10:90, v/v). The results expressed as mean values and recovery are expressed in Table 2. As would generally be expected, recoveries, accuracy and precision improve with increasing antibiotic concentration. The recoveries are particularly significant for the antibiotics that are strongly bound to plasma proteins, such as metronidazole and nafcillin, where the common procedures of solvent extraction and protein precipitation have been shown to lead to variable recoveries in previous studies [20]. These results



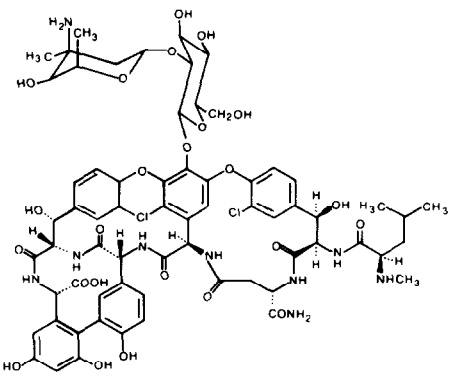
Nystatin $A_1$



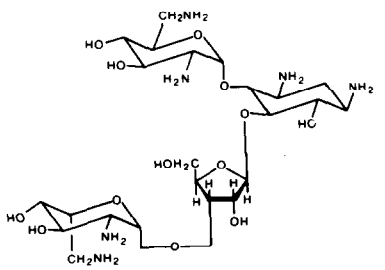
Amphotericin B



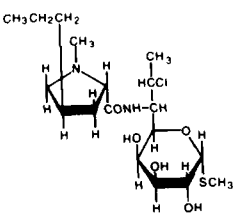
Thiostrepton



Vancomycin



Neomycin B



Clindamycin

indicate that the transfer of antibiotics from the serum protein binding sites to the silica gel resin is rapid. A good correlation was obtained between the pharmacokinetic parameters of area under the curve and maximum serum concentration for metronidazole and nafcillin, as determined by the above direct injection methodology and literature results [21]. Further studies are necessary to explain how the drug protein–drug-silica forces interact.

In addition to the simplification of sample pre-treatment, chromatographic methods using silica columns and aqueous mobile phases are less prone to brand-to-brand and

**Table 1**
Capacity factors of various antibiotics and antimicrobial agents

| Compound | Mobile phase composition 0.1% $H_3PO_4$, pH 2 | 0.1% $H_3PO_4$–$CH_3CN$ (9:1), pH 2 | 0.01 M $NH_4H_2PO_4$, pH 6 |
|---|---|---|---|
| Ampicillin | 11.5 | 7.5 | 10.1 |
| Amoxicillin | 10.0 | 6.0 | 7.3 |
| Cloxacillin | 14.6 | 9.7 | 12.0 |
| Nafcillin | 4.3 | 3.7 | 5.4 |
| Cephradine | 10.5 | 6.4 | 12.1 |
| Cephalexin | 8.1 | 4.9 | 11.2 |
| Cefadroxil | 4.2 | 3.6 | 6.5 |
| Cefotaxime | 18.8 | 12.0 | 14.0 |
| Metronidazole | 2.5 | 1.8 | 2.6 |
| Nystatin | 30 | 7.2 | 5.4 |
| Amphotericin B | 28 | 6.5 | 3.3 |
| Thiostrepton | >30 | 10.4 | 7.4 |
| Vancomycin | 4.5 | 1.0 | 8.4 |
| Clindamycin | 13.0 | 1.0 | 1.8 |
| Sulfamethazine | 4.3 | 3.0 | 3.8 |
| Sulfadiazine | 9.2 | 5.8 | 7.0 |

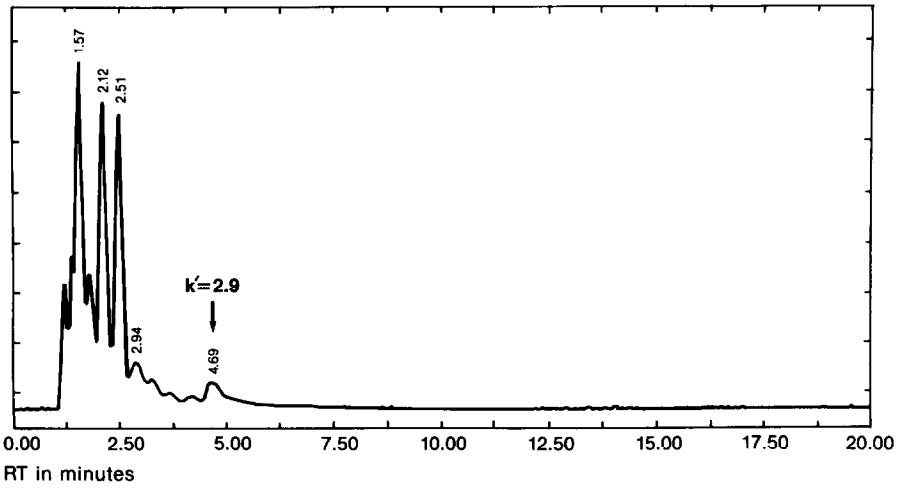



**Figure 2**
Chromatogram of control serum on a silica column monitored at 254 nm with 0.1% phosphoric acid as the eluent and flow rate of 1.0 ml/min. The void volume was estimated by the injection of a 1% acetonitrile solution.

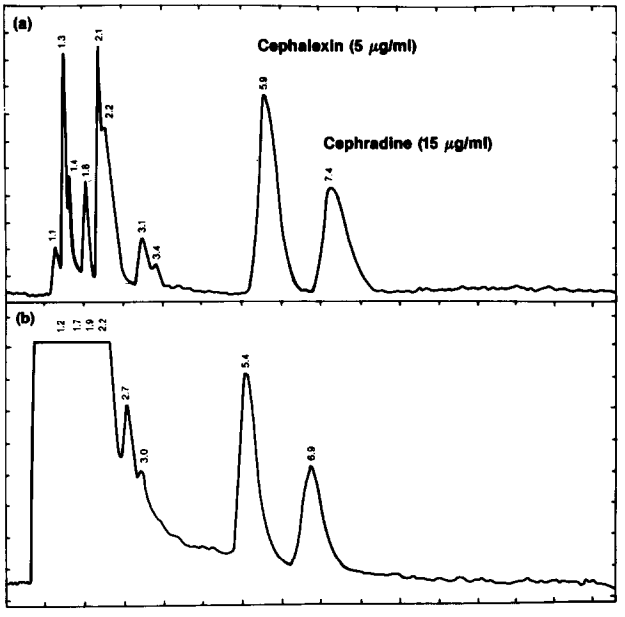



**Figure 3**
Chromatogram of cephalexin and cephradine spiked in control serum, (b) after 50 injections. Mobile phase consisted of 0.1% aqueous phosphoric acid–acetonitrile (90:10, v/v) with monitoring at 220 nm.

**Table 2**
Accuracy, precision and recovery of analysis of four antibiotics at two concentrations spiked in serum

| | Mean (μg/ml) | Recovery (%) | RSD (%) |
|---|---|---|---|
| Serum spiked with 20 μg/ml ($n$ = 5) | | | |
| Cephradine | 20.5 | 102.5 | 3.9 |
| Cephalexin | 20.3 | 101.5 | 3.2 |
| Metronidazole | 20.4 | 102.0 | 1.5 |
| Nafcillin | 19.8 | 99.0 | 4.2 |
| Serum spiked with 100 μg/ml ($n$ = 5) | | | |
| Cephradine | 99.7 | 99.7 | 1.6 |
| Cephalexin | 99.6 | 99.6 | 2.0 |
| Metronidazole | 100.1 | 100.1 | 0.9 |
| Nafcillin | 98.0 | 98.0 | 2.2 |

batch-to-batch variations frequently found when using bonded phase packings [13]. This is an obvious advantage for silica methods since it is important that the same chromatographic conditions for drug analysis give similar results with each new column [2]. Unfortunately, the perception that reversed-phase chromatographic columns are better suited than silica columns for therapeutic drug monitoring is still a commonly held belief [2], even though the use of silica columns with polar eluents was first introduced by Jane [22] in 1975 and has become widely used in forensic laboratories for the screening and quantitative analysis of basic drugs in biological fluids [15]. Furthermore, the

simultaneous determination of serum concentrations of lidocaine and its metabolites on silica with aqueous eluents has been demonstrated [23] along with metoclopramide [24] and alprozolam [25].

An alternative to this approach is the direct serum injection by using micellar liquid chromatography, with aqueous sodium dodecyl sulphate solutions as the mobile phase [26].

In summary, sample preparation of various drugs in serum can be simplified to the direct injection of the sample into a HPLC system through the use of silica-gel columns and essentially aqueous mobile phases. These chromatographic systems can be optimized by varying the pH, buffer and organic content of the mobile phase. Serum protein and fat saturate the silica-gel columns but without deleterious effects upon solute retention or quantification. This approach will be particularly useful for pharmacokinetic studies or therapeutic drug monitoring of solutes not requiring trace enrichment for detection.

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## References

[1] K. C. Cummings, A. R. Torres and S. C. Edberg, *J. Clin. Lab. Automation* **4**, 113–117 (1984).
[2] R. W. Giese, *Clin. Chem.* **29**, 1331–1343 (1983).
[3] I. Nilsson-Ehle, *J. Liq. Chromatogr.* **6**, 251–293 (1983).
[4] A. D. Blair, A. W. Forey, B. T. Meijsen and R. E. Culture, *J. Pharm. Sci.* **63**, 1334–1339 (1975).
[5] K. G. Wahlund, *J. Chromatogr.* **218**, 671–679 (1981).
[6] D. Blair and B. H. Rumack, *Clin. Chem.* **23**, 743–745 (1977).
[7] B. R. Manno, J. E. Manno, C. A. Dempsey and M. A. Wood, *J. Anal. Toxicol.* **5**, 24–28 (1981).
[8] B. R. Manno, J. E. Manno and B. Hilman, *J. Anal. Toxicol.* **3**, 81–86 (1979).
[9] M. W. F. Nielen, E. Sol, R. W. Frei and U. A. Th. Brinkman, *J. Liq. Chromatogr.* **8**, 1053–1070 (1985).
[10] I. H. Hagestam and T. C. Pinkerton, *Anal. Chem.* **57**, 1757–1763 (1985).
[11] B. Henke and D. Westerlund, *J. Chromatogr.* **187**, 189–198 (1980).
[12] D. Westerlund and K. H. Karset, *Anal. Chim. Acta.* **67**, 99 (1973).
[13] J. Adamovics, *J. Liq. Chromatogr.* **2**, 393–396 (1984).
[14] B. A. Bidlingmeyer, J. K. Del Rios and J. Korpi, *Anal. Chem.* **54**, 442–447 (1982).
[15] B. Law, R. Gill and A. C. Moffat, *J. Chromatogr.* **301**, 165–172 (1984).
[16] S. H. Hansen, P. Helboe and M. Thomsen, *J. Pharm. Biomed. Anal.* **2**, 165–172 (1984).
[17] Y. I. Yashin, *Chromatographia* **16**, 318 (1983).
[18] T. Arvidsson, K.-G. Wahlund and N. Daoud, *J. Chromatogr.* **317**, 213–226 (1984).
[19] M. Simonyi, *Med. Res. Rev.* **4**, 359–413 (1984).
[20] J. T. Rudnik and R. E. Bawdon, *J. Liq. Chromatogr.* **4**, 1525–1545 (1981).
[21] W. A. Creasey, J. Adamovics, R. Dhruv, T. Platt and A. Sugerman, *J. Clin. Pharmacol.* **24**, 174–180 (1984).
[22] I. Jane, *J. Chromatogr.* **111**, 227–234 (1975).
[23] K. Kushida, K. Oka, T. Suganuma and T. Ishizaki, *Clin. Chem.* **30**, 637–640 (1984).
[24] R. J. Y. Shi, P. R. B. Wang, R. L. Williams, L. Z. Benet and E. T. Lin, *American Pharmaceutical Association Academy of Pharmaceutical Sciences 39th National Meeting* **15**, 130 (1985).
[25] W. J. Adams, P. B. Bombardt and J. E. Brewer, *Anal. Chem.* **56**, 1590–1594 (1984).
[26] F. J. DeLuccia, M. Arunyanart and L. J. Cline Love, *Anal. Chem.* **57**, 1564–1568 (1985).

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