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Review

Chromatographic analysis of antibiotic materials in food

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ABSTRACT

The monitoring of food materials for antibiotic residues is an area of increasing concern and importance due to the potential impact on human health. Large-scale screening applications require methods that are rapid, accurate, provide low detection limits and are free from interference. The problem is further complicated by the wide range of chemical functionalities and modes of operation exhibited by the antibiotic materials of physiological significance in use today. As demonstrated, chromatographic methods provide many of the advantages necessary for screening applications. Judicious choice of sample preparation method, separation mode and detection strategy can provide significant immunity from problems associated with the food matrix.

Gas chromatography can provide extremely high separation efficiencies, however, only a limited number of antibiotic compounds are inherently volatile enough for direct analysis by gas chromatography. Derivatization to enhance the volatility of the antibiotic is one approach to overcome this limitation. Among the methods available, reversed-phase high-performance liquid chromatography is used extensively for the analysis of many antibiotic systems as it does not require derivatization and it combines relatively high separation efficiencies with low detection limits. The diverse group of properties exhibited by the antibiotic materials in use today suggests that the choice of detection strategy is a key component in the successful development of an analysis technique. Derivatization of the antibiotic material is frequently used to add either a fluorogenic of chromogenic moiety to the antibiotic compound to enhance detection. Derivatization procedures suffer from several limitations which are problematic when making measurements in complicated food matrices. Among the different detection modes ultilized for antibiotic analysis, polarimetric detection has the potential to provide extremely selective detection of most antibiotic materials, and this selective response can minimize many of the constraints placed upon the separation system by the sample matrix. Although many of the separation modes used for antibiotic analysis are well developed, separations based on capillary electrophoretic methods have much potential in the field of antibiotic analysis. Future investigations are needed to extend the generality of these techniques and expand their use into the field of food analysis.

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1. INTRODUCTION

Antibiotics are an extremely important class of compounds as they represent a key component in the strategy used to control bacterial infections in both human and animals. Chemically, the collection of materials which exhibit antibiotic properties are a diverse group with widely divergent chemical functionalities and modes of operation. This diversity, though, presents a tremendous challenge to the analyst as subtle structural variations in closely related antibiotic materials can lead to pronounced differences in the chemical toxicity and biological activity of the antibiotic. Thus there is the need to develop rapid analytical methods for antibiotic materials which could be used to screen suspect products for their presence.

Traditional microbiological assays, that is, those methods that involve the growth of a probe microorganism on a medium containing the antibiotic, suffer from a variety of limitations including the time and labor-intensive nature of the procedure. In addition, microbiological assays cannot differentiate among the various forms and derivatives of a given antibiotic system, and the quantitative information offered by such an approach reflects the total amount of all forms of a given antibiotic, rather than providing distinct quantitative information on different analogues. This information can be very useful as it will be shown that the relative distribution of various analogues in a given antibiotic system is unique and can be indicative of the source of the particular antibiotic sample. Thus, knowledge of the distribution of components can be useful from both a physiological perspective, that is, one analogue usually has minimal toxicity and maximum antibiotic activity, and for recognizing the source of contamination.

In contrast to microbiological methods, chromatographic approaches can provide a rapid response and offer both high sensitivity and separation efficiencies. Thus, chromatographic methods have the potential to provide many of the characteristics necessary for systematic screening of food materials. However, the extremely diverse nature of antibiotic materials requires that a variety of different separation modes, detection strategies and sample preparation procedures be used to achieve the goals outlined previously as necessary for rapid and sensitive screening. Several recent reviews have appeared that describe various chromatographic strategies for the separation of antibiotic materials found in food materials [1–3]. These reviews are limited in their scope and application as they key either on a specific antibiotic system or on only a few antibiotics. Ref. 1 provides an excellent summary of extraction/deproteinization systems used for isolating a variety of antibiotic materials found in different food matrices.

This work will focus on chromatographic methods for separating antibiotic compounds found in food materials. Methods selected for inclusion have demonstrated enhanced selectivity towards the various structural analogues in a given system, and, in many cases, the high separation power of the chromatographic system minimizes the amount of sample preparation required. Novel detection strategies will also be discussed which can impart additional selectivity to the method. Finally, problems inherent to antibiotic analysis in specific food matrices will be presented with the goal to highlight those aspects of the separation system that are necessary to minimize, or eliminate, interferences from the matrix.

2. SEPARATION MODES FOR ANTIBIOTIC ISOLATION AND ANALYSIS

2.1. Gas chromatography

Gas chromatography (GC) possesses the requisite chromatographic resolution to separate closely related antibiotic analogues. However, most antibiotic materials of physiological significance are not sufficiently volatile for direct GC analysis. In situations where the antibiotic material is present at extremely low levels, the high sensitivity of the electron-capture or thermionic detector may be advantageous and justify the time necessary to derivatize the antibiotic to enhance its volatility. For example, chloramphenicol is a highly regulated antibiotic which may be present in food materials at extremely low levels and which can severely impact human health in susceptible individuals at these levels. Allen [2] has reviewed eight different GC methods in which chloramphenicol is derivatized using either trimethylsilane (TMS), hexafluorobutylacetate (HFBA) or ethyl acetate [4]. Electron-capture detection provides a limit of detection (LOD) at the 1–10 ng/g level in most food matrices with recoveries ranging from 72% (unpolished rice) to >90% in milk and meat. In general the added steps necessary for derivatization are problematic and methods based on liquid chromatography (LC) have received much recent interest.

2.2. Thin-layer chromatography

Thin-layer chromatographic (TLC) methods can provide high sample throughput which is advantageous in screening applications. In general, the method has been limited in the past by poor chromatographic resolution, minimal selectivity and high detection limits. Oka and co-workers [5,6] have demonstrated that high-performance TLC (HPTLC) can solve several of these problems. As is evident from Fig. 1, six of the main tetracycline analogues can be separated by HPTLC with excellent chromatographic resolution. Reversed-phase TLC (RP-TLC) (C_8) was used to alter the elution order to aid in identification of the different analogues

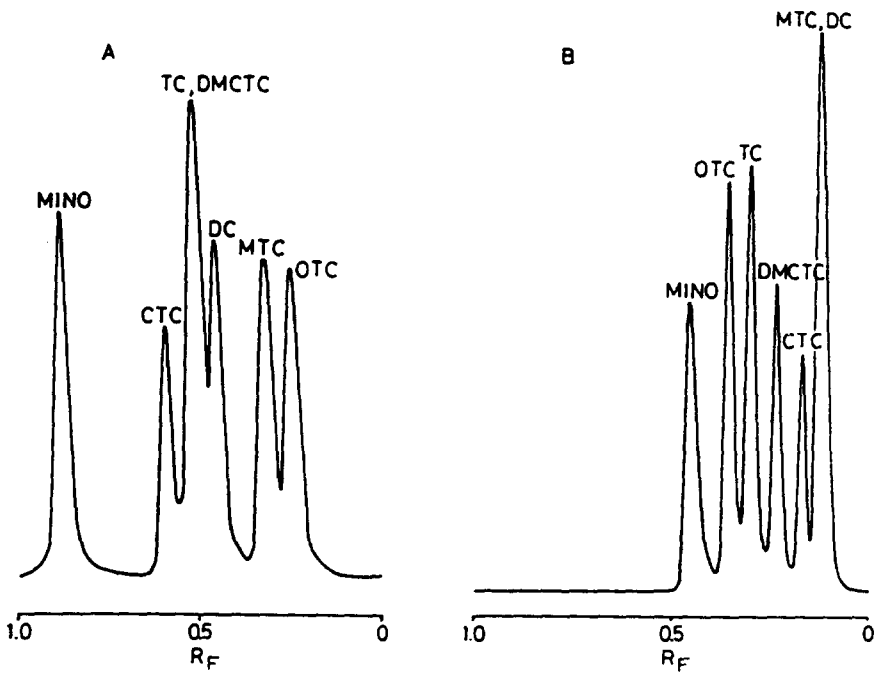

Fig. 1. Elution profiles for selected tetracyclines separated by HPTLC. (A) Pre-developed silica gel HPTLC plate saturated with Na_2EDTA and activated for 2 h at 130°C. Solvent system: chloroform–methanol–5% Na_2EDTA (65:20:15). (B) RP-TLC plates. Solvent system: methanol–acetonitrile–0.5 *M* oxalic acid (1:1:4), pH 3.0. From ref. 5.

and minimize possible interferences from the food matrix. Detection requires either the addition of a visualization agent (Fast Violet B Salt) to the plate with heating to create a colored product, or direct ultraviolet (UV) densitometry can be used. Visualization agents provide a rapid response but are only semi-quantitative in their response.

2.3. Liquid chromatography

2.3.1. Reversed-phase chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) combines rapid sample throughput, high sensitivity (detection mode-dependent) and high chromatographic selectivity. However, the expense of efficient HPLC columns and the ease with which they can be damaged suggest that it would be prudent to pretreat food extracts to limit irreversible adsorption of highly hydrophobic materials on the column. For example, both sulfonamides [7] and tetracyclines [8] have been extracted from animal tissues under acidic conditions and the extractants cleaned up by the use of a Sep-Pak C_{18} cartridge. Sulfonamides were eluted from the cartridge with methanol, while the tetracyclines were eluted using a mixture of dimethylformaldehyde (DMF)–water (4:6). The eluted antibiotic mixtures were then separated into their respective structural analogues by RP-HPLC. With the Sep-Pak treatment the recovery of the sulfonamides ranged from 77 to 103%, however, tretracycline recovery was less than 60% for most animal tissues.

The separation power of HPLC with respect to the differentiation of closely related structural analogues of antibiotic materials is evident in Fig. 2 [9]. In Fig. 2, RP-HPLC is used to separate ampicillin and the (5*R*,6*R*)- and (5*S*,6*R*)-epimers of penicilloic acid. These epimeric forms of penicilloic acid have identical molecular masses but differ solely by the arrangement of atoms at one chiral center in the molecule. Near-baseline resolution is obtained for the epimeric pair allowing accurate and precise quantitation of each of the compounds.

In situations where more detailed structural analysis of the separated antibiotics is required for precise identification, HPLC separation has been combined with mass spectrometric (MS) detection. However, the vacuum requirements of the mass spectrometer are incompatible with the solvent throughput characteristic of conventional HPLC columns. Moore *et al.* [10] have solved this problem by using 150 mm × 0.3 mm I.D. microbore columns. The analytical column was preceded by a 30 mm × 0.3 mm I.D. guard column. The use of the microbore column reduced the mobile phase flow-rate by a factor of 200 which allowed direct connection of the column to the mass spectrometer. The authors avoided mobile phases containing salts which easily crystallize (*e.g.* phosphate) to minimize column blockage. The combined HPLC–MS system provided efficient detection and identification of cephalosporins at the 1–5 ng level.

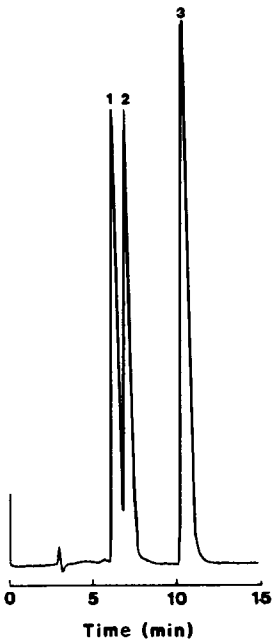

Fig. 2. Separation of (5*S*,6*R*)- (1) and (5*R*,6*R*)- (2) epimers of penicilloic acid and ampicillin (ABPC) (3). Concentrations of the 5*S*,6*R*- and 5*R*,6*R*-epimers were approximately 10 μg/ml, that of ABPC 12.5 μg/ml. Injection volume: 20 μl. Eluent: 20 m*M* phosphate buffer (pH 4.2)–methanol (3:1, v/v) at a flow-rate of 0.8 ml/min. Absorption detection (290 nm) at a sensitivity of 0.04 a.u.f.s. Reaction coil length was 2 m. From ref. 9.

2.3.2. Ion-pair chromatography

Many antibiotic systems possess functional groups which are ionized at pH values compatible with silica-based RP-HPLC stationary phases. Therefore, the formation of an ion pair with the

antibiotic material can produce very efficient separations with additional modes of chromatographic control. Mobile phase pH (which controls the extent of ionization), the type of counter ion (which governs the interaction with the stationary phase and formation constant of the ion pair) and the concentration of the ion-pair reagent (to drive the equilibrium) can all be varied to control the separation. Yoneda *et al.* [11] has used 0.2 *M* HCl as an eluent to separate aminoglycoside antibiotics. This aggressive mobile phase did not inhibit the post-column detection reaction with *o*-phthalaldehyde and recoveries from beef were of the order of 80% with detection limits at the 0.2-μg/g level. Voydsner *et al.* [12] utilized a variety of alkylsulfonates to achieve efficient separation of several penicillin derivatives extracted from bovine milk and separated on a 2.1 mm I.D. column. Both C_8 and C_{12} alkylsulfonates were used, either separately or in combination. The choice of both ion-pair reagent and concentration was found to be useful in enhancing the separation efficiency and in minimizing interference from co-extracted components of the milk. The alkylsulfonates did not affect the detection mode as both UV–photodiode array (PDA) and thermospray MS were used. The UV–PDA system gave slightly better detection limits, but the mass spectrometer produced both $[M-H]^+$ and $[M-Na]^+$ ions which provided unambiguous identification of each penicillin analogue.

2.3.3. Immunoaffinity chromatography

An innovative approach to minimize the matrix effect of samples such as eggs and milk has been demonstrated by Van de Water *et al.* [13]. An antibody-mediated clean-up routine has been used to provide excellent recovery of chloramphenicol from these samples. In this approach, a monoclonal antibody to chloramphenicol was immobilized on a carbonyldiimidazole-activated support. The high specificity of the interaction allowed large samples to be processed and concentrated. Conventional HPLC was used to access residual matrix effects after the immunoaffinity column clean-up. Fig. 3 shows the results of this study. The immunoaffinity column is extremely selective and, as is evident, no extraneous peaks were observed within the elution window for chloramphenicol that would interfere with quantitation. This was true for both the egg and milk samples. The column was very durable when the antibiotic was eluted with a mixture of glycine (0.2 *M*) and NaCl (0.5 *M*, pH 2.8) and a single column was reused over 30 times. However, it was also found that recoveries with the immunoaffinity column were lower than those obtained using a conventional solid-phase extraction procedure. One possibility is that the chloramphenicol is protein-bound and either lost in the precipitation step, or the protein complex cannot be bound by the immunoaffinity column. Further study is necessary to understand and control this limitation.

2.4. Supercritical fluid chromatography

Packed-column supercritical fluid chromatography (SFC) with UV detection has been evaluated as a separation technique for the analysis of sulfonamides found in swine kidney extracts [14]. Theoretically, SFC maintains much of the separation power available in capillary column GC without requiring that analytes be volatile and thermally stable. The mobile phase consisted of supercritical carbon dioxide with varying concentrations of methanol as a modifier. Other modifiers were tested (*e.g.* DMF) but were found to be of limited use in modifying the chromatographic properties of the mobile phase. Both silica and amino-bonded stationary phase columns were used and each demonstrated distinctly different selectivities with respect to the family of sulfonamides. It was also observed that mobile phase modification with methanol provided different effects with the two columns in that the amino-bonded phase was much more sensitive to modifier variations. The authors thus suggest that the differing selectivities observed for the different stationary phases and modifier concentrations can be used to choose a specific separation system for a specific problem.

2.5. Capillary electrophoresis

Capillary electrophoretic (CE) methods utilize a small-diameter silica capillary (25–75 μm) as the separation vessel with applied field strengths that approach 500 V/cm to separate ionic materials. Much recent work has focused on the high separation efficiencies possible with this separation mode and 10^6 plates/m have been demonstrated. Ideally,

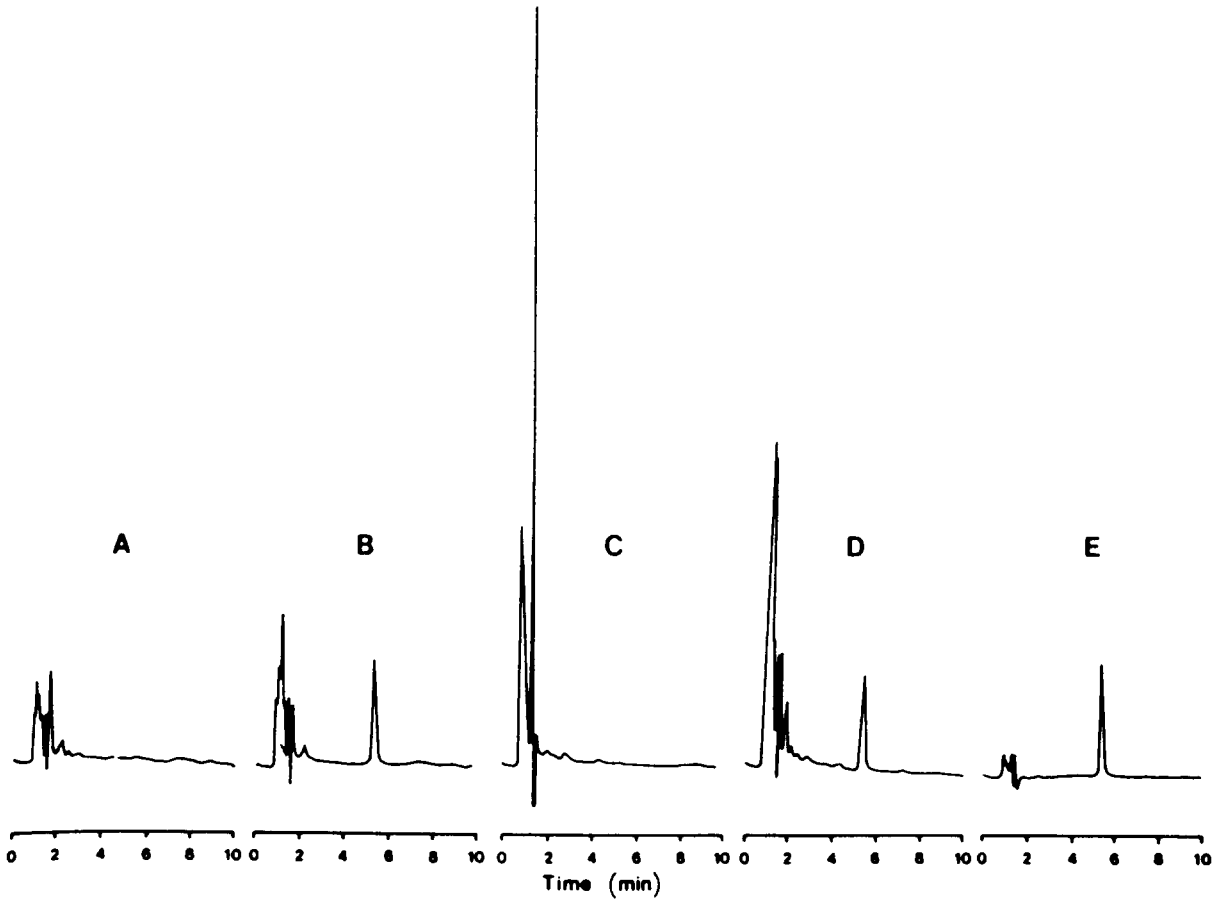

Fig. 3. HPLC analysis of chloramphenicol (CAP) obtained from milk and egg samples and purified by means of antibody-mediated clean-up. (A) Blank milk sample; (B) spiked (10 μg/kg) milk sample; (C) blank egg sample; (D) spiked (10 μg/kg) egg sample; (E) standard solution of CAP. Absorbance detection at 0.016 a.u.f.s. From ref. 13.

these characteristics make CE particularly well suited for the separation and analysis of antibiotic materials found in food matrices. However, many antibiotic compounds are neutral, or only minimally ionized over large pH ranges, and therefore not directly amenable to the conventional CE technique.

Nishi and co-workers [15,16] utilized micellar electrokinetic chromatography (MEKC) to separate both penicillin and cephalosporin antibiotics. In this mode, sodium dodecyl sulfate (SDS), a surfactant, is added to the electrophoretic buffer at a level above the critical micelle concentration (CMC). The micelle phase provides a new avenue of selectivity as the neutral antibiotics can differentially partition into the interior of the micelle. Migration in MEKC for these antibiotic materials was found to depend on the inherent electrophoretic mobility of the analyte, the distribution of the analyte in the micellar phase and the ability of the analyte to ion pair with the micelle. In this mode, anionic, cationic and neutral or zwitterionic materials can be separated in a single system. Fig. 4 shows a CE and MEKC separation of nine cephalosporin antibiotics. The high separation efficiency available with this technique is clearly evident in the three electropherograms. The extension from the CE to the MEKC mode (A to B) shows the enhanced selectivity available with the micelle phase. C shows

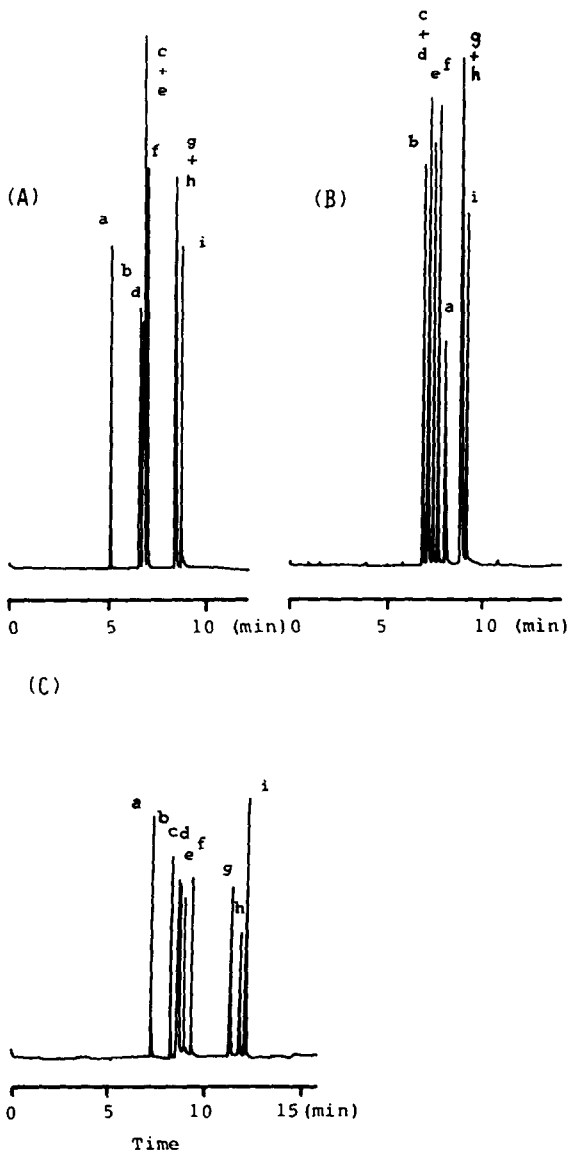

Fig. 4. Effect of tetraalkylammonium salts (TAA) on the micellar electrokinetic chromatographic (MEKC) separation of nine cephalosporin antibiotics: (a) C-TA; (b) ceftazidime; (c) cefotaxime; (d) cefmenoxime; (e) cefoperazone; (f) cefpiramide; (g) cefpimizole; (h) cefminox; (i) ceftriazone; (A) CZE mode with 0.02 *M* phosphate–borate buffer (pH 9.0); (B) MEKC mode with 0.02 *M* phosphate–borate buffer (pH 9.0) containing 0.05 *M* SDS (sodium dodecyl sulfate); (C) MEKC mode with 0.04 *M* TMAB (tetramethylammonium bromide) added to the same SDS solution as in (B). Conditions: buffer, 0.05 *M* acetate buffer; applied voltage, 20 kV; temperature, ambient; detection wavelength, 210 nm. From ref. 16.

the dramatic effect the addition of tetraalkylammonium (TAA) salts to the CE buffer can have as all nine antibiotics are separated in a single run. It was suggested that the TAA salts form ion pairs with anionic materials, and the partitioning of the neutral ion pair into the micelle is increased thereby increasing the retention of the solute.

Another approach is to use capillary isotachophoretic chromatography to separate penicillin and cephalosporin antibiotics and their precursers [17]. Judicious choice of the leading buffer system can be used to separate the various analogues of a given antibiotic. In general, these reports demonstrate the large potential CE has with respect to the analysis of antibiotic materials in food. High separation efficiencies and multiple separation modes and mechanisms provide a number of different strategies which can be used to analyze antibiotics in complicated matrices. It is clear that this potential will drive the technology into new areas and applications which have proven difficult to solve by conventional chromatographic approaches. The present limitation to the application of this technology is the need to provide more reproducible sample introduction and detection techniques.

3. DETECTION MODES TO ENHANCE SELECTIVITY AND SENSITIVITY

3.1. General overview

The diverse group of properties exhibited by the pharmacologically significant antibiotics makes both their separation and detection difficult. Several antibiotic systems possess inherent properties which facilitate detection. Chloramphenicol, for example, exhibits strong absorption above 260 nm as a consequence of its aromatic structure. However, in most cases, alternative strategies have to be applied in order to enhance detection sensitivity due to the low residual levels at which many of these materials must be monitored. The problem is further compounded by the fact that a detection strategy that works for one antibiotic system may not work for another. Yet in many treatment regimes, two or more antibiotics may be used simultaneously to extend the range of susceptible organisms. There have been several reports of detection systems which are universal in their response. However, in the case of

MS, cost, sensitivity and limitations due to co-eluting materials have precluded its adoption for routine applications. As will be discussed, polarimetric detection is a possible detection strategy which is almost universal in its response to antibiotic materials.

Very few antibiotic systems possess chromophores which are accessible above 230 nm. Alternatively, one can use short-wavelength UV detection, however, in this spectral region, many materials absorb appreciably and chromatographic mobile phases must be chosen with respect to both their chromatographic and spectroscopic properties. For example, Terada and Sakabe [18] combined ion-pair chromatography with UV detection at 210 nm to separate and analyze penicillins found in milk. A Sep-Pak clean-up procedure proceeded the chromatographic analysis. However, even with this procedure, many materials were detected during the elution window of the three penicillins tested. In order to differentiate the antibiotic materials from other components of the milk sample, penicillinase was added to degrade the penicillins. Subsequent chromatographic analysis of the degraded sample and comparison with the original chromatogram showed the absence of three peaks which corresponded to the penicillins. In general, for routine screening of widely diverse samples, short-wavelength UV detection is not sufficiently selective. The complicated nature of the chromatographic data obtained in this mode will make low-level identification and quantitation difficult, or impossible.

3.2. Derivatization to enhance detection

Chemical transformation of an antibiotic substance through the addition of either a chromophore or a fluorophore can be used as a detection strategy to enhance the materials detectability. This approach adds time, complexity and cost constraints to the procedure. For pre-column derivatization, the chromatographic resolution of closely related substances can be jeopardized as the added moiety may dominate the chromatographic properties of the derivative. Thus subtle variations in the chromatographic properties within a group of related analogues may be lost through the derivatization procedure. Further, derivative stability will be a main consideration when choosing the appropriate analytical procedures to use in a given situation. Post-column derivatization does not directly affect the chromatographic properties of the antibiotic, however, the reaction chemistry must be rapid on the chromatographic time scale in order to preserve the chromatographic information. Further, the post-column reaction chemistry may be affected by the chromatographic conditions and this could limit the types of mobile phases that may be used.

Pre-column derivatization procedures have been developed for a number of antibiotic systems and several representative examples are summarized in Table 1. In general, chemical derivatization becomes more difficult to implement as the complexity of the sample matrix increases.

TABLE 1

SELECTED PRE-COLUMN DERIVATIZATION AGENTS FOR ANTIBIOTIC MATERIALS

Antibiotic	Derivatizing agent	Detection mode	Ref.
Erythromycin	Disodium-2-(stilbyl-4″)-4-(naphthol-1′,2′:4,5)-1,2,3-triazole 2″-6′-disulfonate	Absorbance	19
	Ethylsuccinyl chloride	Fluorescence	20
Penicillins	4-Bromethyl-7-methoxycoumarin	Fluorescence	21
Gentamicin	2,4-Dinitrophenyl derivatives	Absorbance	22
	1-Fluoro-2,4-dinitrobenzenen	Absorbance	23
β-Lactams	Imidazole–metal salt	Absorbance	24
Mononsin	9-Anthryldiazomethane	Fluorescence	25
Inophores	9-Anthryldiazomethane	Fluorescence	26
Sulfonamides	Fluorescamine	Fluorescence	27
Fortimicin A	3,5-Dinitrobenzoyl Chloride	Absorbance	28

3.3. Polarimetric detection

Most antibiotic materials and their derivatives possess the property of chirality, that is, they are optically active. This is a consequence of the fact that they are produced by living organisms which use chirality as part of the system of molecular recognition. Therefore, optical activity is an extremely rare characteristic and it is usually associated with biological activity, past or present. The application of optical activity detection to the analysis of antibiotic materials should provide many of the advantages characteristic of the ideal detection system. Assuming that the sensitivity of the optical activity detection system is sufficient to handle the extremely dilute conditions encountered in HPLC, the detector would provide significant specificity without requiring derivatization prior to detection. Thus the separation system could be chosen with regard only to the separation problem.

Several recent reports have described the application of optical activity detection to the study of antibiotic materials after separation by HPLC. The laser-based polarimeter used for these studies utilizes a helium–neon laser (He–Ne) to achieve rotational detectabilities at the 5–10 μdegree level [29]. The instrumentation uses relatively unsophisticated components, and commercial polarimetric detection systems are now available.

Specific rotation, $[\alpha]$, is the physical parameter used to describe the innate optical activity of a substance. Since optical activity is such a rare property, specific rotation measurements on eluting materials could have potential in identifying closely related structural analogues in a given antibiotic system.

Laser-based polarimetric detection has been applied to the study of erythromycin in milk [30]. Several significant results were inferred from this work. First, as expected, polarimetric detection provided a selective response which removed some of the constraints placed upon the separation system by the complicated milk matrix. The milk sample was extracted with a 4:1 mixture of pentanol and chloroform and the extractant injected directly into the HPLC system. The polarimetric detection system provided significant specificity as only a few peaks, in addition to those due to erythromycin, were observed. Refractive index detection of the same solution showed that there were many more components eluting from the HPLC column. Further, the polarimetric system was very sensitive providing minimum detectable quantities at the 10-ng level for erythromycin. Finally, and most importantly, specific rotation measurements were made on the antibiotic materials as they eluted from the HPLC column. Commercial erythromycin preparations consist of three closely related analogues designated as erythromycins A, B and C. The specific rotation obtained for erythromycins A and C differed by almost 10% even though the structural variation which differentiates these two analogues consists of the substitution of a hydroxyl (C) for a methoxy (A) group. This difference entails a mass difference of approximately 2%. Thus, specific rotation measurements have potential in identifying closely related structural analogues when studying their fate or location in complex physiological pathways or biological matrices.

The sensitivity of specific rotation to subtle structural variations was tested in the analysis of the epimers of ticarcillin and carbenicillin [31], two members of the penicillin family of antibiotics. Each epimer contains a total of four chiral centers, but they differ by the arrangement of atoms at a single one of these centers. Polarimetric detection of these epimers after separation by HPLC provided detection at the 10-ng level. More importantly, specific rotations were obtained for each epimer as it eluted from the separation system. The results of these measurements are summarized in Table 2 below. The large difference in specific rotation measured for the epimeric forms of these two materials is very surprising given the minor structural variation which differentiates the two forms. Clearly, polarimetric detection of antibiotic materials has demonstrated significant advantages from both a qualitative and a quantitative perspective when compared to other detection methods used for these materials.

As seen in the erythromycin study discussed previously, organisms which produce antibiotic substances frequently produce several closely related structural analogues. The relative abundance of these different analogues can vary depending upon the conditions used during the growth phase of the organism. Thus, the relative distribution of the different analogues in a given system can be indicative of the source of that antibiotic sample and this information may be useful for identification.

TABLE 2

SPECIFIC ROTATION MEASUREMENTS FOR SIX PENICILLIN ANALOGUES

Compound	Specific rotation (deg $dm^{-1}g^{-1}$ ml)		
	Literature	Direct measurement[a,b]	Calculated from peak height[a,b]
Penicillin G	310	340 ± 11	347 ± 6
Ampicillin	281	314 ± 11	321 ± 6
(10*R*)-Carbenicillin	–	192 ± 8[c]	180 ± 1
(10*S*)-Carbenicillin	–		213 ± 1
(10*R*)-Ticarcillin	–	179 ± 8[c]	166 ± 1
(10*S*)-Ticarcillin	–		210 ± 2

[a] 632.8 nm.
[b] Experimentally determined standard deviations.
[c] Epimeric mixture.

This hypothesis was recently tested for gentamicin in which reversed-phase ion-pair chromatography, in combination with laser-based polarimetric detection, was used to analyze milk samples containing this antibiotic materials [32]. A representative chromatogram for gentamicin samples obtained from two different sources is given in Fig. 5. Gentamicin does not possess a chromophore which is accessible above 230 nm. Therefore, previous studies have utilized chemical derivatization of the gentamicin to render it either fluorescent or absorbing. However, the derivatized gentamicin analogues were difficult to separate as the subtle differences which distinguish the four analogues were lost and under optimum conditions only three of the four main analogues were resolved. The polarimetric detection system does not require derivatization prior to detection, and separation of the four main components was now possible. Finally, with all four components resolved, it is evident that the two gentamicin samples contain different amounts of the four analogues and this information may be of use in identifying the source of the sample.

This hypothesis was tested using a milk sample spiked with gentamicin. The chromatogram presented in Fig. 6 shows the reversed-phase ion-pair

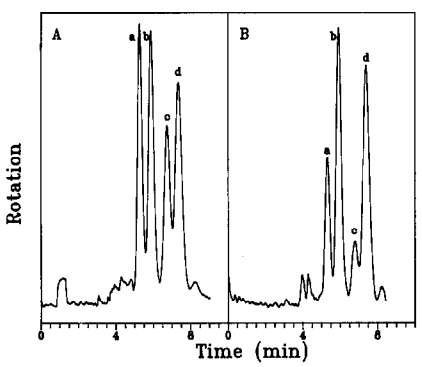

Fig. 5. Laser-based polarimetric detection of gentamicin analogues from two commercial gentamicin preparations after separation by reversed-phase ion-pair chromatography. (A) Supplier 1; (B) supplier 2. Total amount injected was 10 μg. Elution order: a, C_{1a}; b, C_2; c, C_{2a}; d, C_1. Eluent: methanol–0.4 *M* TFA in water (20:80, v/v); flow-rate: 0.75 ml/min. The disturbance in chromatogram A at approximately 1 min is the standard signal produced by a DC Faraday coil corresponding to a rotation of $2.86 \cdot 10^{-4}$ deg. From ref. 32.

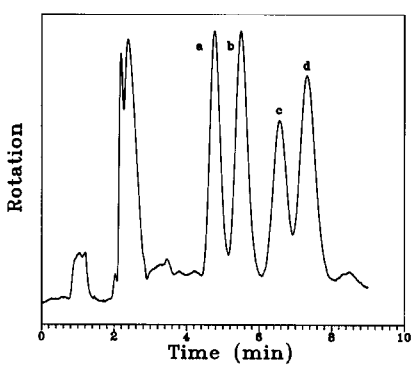

Fig. 6. Separation and detection of gentamicin analogues extracted from milk. Experimental conditions same as in Fig. 5. From ref. 32.

separation of gentamicin analogues obtained from a milk sample. The selectivity of the polarimetric system is a distinct advantage when working with complicated food matrices. Each milk sample was acidified with trifluoroacetic acid (TFA), centrifuged for 10–15 min, and the supernatant liquid was then injected directly into the HPLC column without further preparation. Thus, including the chromatographic run, less than 25 min per sample are required for analysis. Comparison of Figs. 5 and 6 clearly shows the source of the gentamicin sample used to spike the milk.

Polarimetric detection, in combination with HPLC separation, has demonstrated significant advantages for the analysis of optically active antibiotic compounds found in food materials. The approach is fairly universal for most pharmacologically important antibiotics and sensitive detection can be achieved without the need to derivatize the antibiotic. Non-derivatized materials are easier to resolve chromatographically. The polarimetric detection system can provide information on the innate optical activity of eluting antibiotics and this information cannot be obtained by any other means. Specific rotations can be used to identify closely related structural analogues even when present in a complicated food matrix.

3.4. Mass spectrometry

MS is another detection mode that can theoretically provide detection for all antibiotic substances. Two limitations preclude widespread utilization of this detection mode for screening applications; the cost and complexity of the instrumentation, particularly LC–MS, and the difficulty in obtaining diagnostic molecular ions which can be used for both identification and quantitation. A variety of different ionization modes have therefore been used to provide reproducible ion formation. In general, the mass spectral information is usually used for qualitative purposes, while a separate UV detector is then used in-line in order to provide enhanced quantitative information.

Sulfonamides have been determined in salmon flesh using ion-spray tandem MS (MS–MS) [33]. The MS–MS technique, using collision-induced dissociation, provided unique structural information which was used to distinguish between isomeric and isobaric sulfonamides. The MS information was very useful for identification purposes, however, UV PDA detection was used to provide a limit of detection at the 25-ng/g level.

Thermospray (TSP) MS was used to study gentamicin analogues separated by reversed-phase ion pair HPLC using TFA as the ion-pair reagent [34]. Fragmentation patterns were used to identify the four main components. Several previous studies utilized alkylsulfonates as ion-pair reagents for gentamicin separation. However, it was found that these salts were not compatible with the TSP source and TFA was therefore used. Detection limits for the HPLC–TSP-MS technique were approximately an order of magnitude higher than those reported using fluorescent derivatization.

Particle beam HPLC–MS has been evaluated as a potential method for the analysis of antibiotic compounds found in food materials [35]. The sensitivity of the particle beam interface was found to be related to the heat capacity of the solvent system with the highest sensitivity observed for solvents with the lowest heat capacities (*i.e.* methanol > acetonitrile > water). This would limit to some extent the range of mobile phase compositions available for use with this detection system. A LOD at the ng/g level was demonstrated under single-ion monitoring conditions. The particle beam system was also compared to the TSP source. For the same antibacterial compounds, the TSP source provided less structural information than the particle beam, however, the LOD for the particle beam was approximately a factor of 10 higher.

For antibiotic materials that are either inherently volatile enough for GC or derivatizable to enhance their volatility, GC–MS can provide all of the qualitative advantages of LC–MS, with much better LODs. For example, GC–MS analysis of egg samples for chloramphenicol residues [36] using negative-ion chemical ionization provided an LOD which was as good as that reported for the electron-capture detector [37] in a similar matrix.

3.5. Miscellaneous detection techniques

Several new detection modes have been reported for specific antibiotic materials. These include electrocatalytic oxidation [38], tris-2,2′-bipyridyl ruthenium(III)-based chemiluminescence [39] and chem-

ical degradation to produce an absorbing product [9]. These systems are narrow in their application and do not provide the unique information characteristic of the polarimetric or MS techniques. In addition, these methods have not demonstrated detection limits which are significantly better than the other approaches described and they therefore have not been widely applied to the analysis of food materials.

4. CHROMATOGRAPHIC ANALYSIS OF ANTIBIOTICS IN SPECIFIC FOOD MATRICES

4.1. Fish and meat

For routine screening of antibiotic residues in animal tissues, the analytical procedure utilized should provide a rapid response, it should be relatively immune from interference, and for low level monitoring it should provide low detection limits and be able to discriminate among several close structural analogues within an antibiotic class. Thus the general strategy is to extract the antibiotic from the tissue sample, isolate the antibiotic from other, co-extracted materials (proteins, fats, etc.) by either additional solvent extractions or by the use of a solid-phase extraction technique, and then use HPLC to separate and detect the various analogues within a given antibiotic system.

Interferences arise primarily from the characteristics of the food matrix. For example, animal tissues contain large amounts of protein which usually requires considerable clean-up prior to chromatographic analysis. Since many of the antibiotic materials used in veterinary applications are hydrophobic, most methods developed for use with animal tissues utilize reversed-phase cartridges for sample clean-up. However, elution of the antibiotic materials from the cartridge requires organic solvents which must be eliminated prior to chromatographic analysis. Further, reversed-phase cartridges will extract other lipophyllic materials in addition to the antibiotic compound and these must be removed in a separate procedure prior to analysis. Ikai *et al.* [40] used an amino-type prepacked cartridge for the clean-up of sulfonamide antibiotics present in animal tissues. The use of an amino cartridge significantly shortened the sample preparation time. Tissue samples were extracted with ethyl acetate and the extractant then passed through the amino cartridge. Lipophillic materials were removed from the cartridge with several hexane washes, and the sulfonamides were eluted with a 24:76 acetonitrile–0.2 *M* phosphoric acid solution. The antibiotic materials were then separated and detected using a reversed-phase column with UV detection at 272 nm.

Fig. 7 shows the reversed-phase separation of a series of ten sulfonamides separated from commercially available meats, eel and egg. The amino cartridge procedure provides effective isolation of the sulfonamide compounds resulting in a wide elution window. The complete procedure required approximately 45 min per sample. Recoveries for these antibiotic materials were in the range 74–99% with a demonstrated detection limit at the 50-ng/g level. The advantages of this procedure in terms of minimal sample preparation are not limited solely to sulfonamides but should be applicable to other antibiotic systems of similar hydrophobicity.

Wulders and Van de Lagemaat [41] have developed a Sep-Pak cartridge-based method for use in isolating tetracycline antibiotics from animal tissues. The reversed-phase nature of the Sep-Pak requires additional sample preparation and manipulation as cited earlier. Specifically, prior to chromatographic analysis, tetracyclines were eluted from the Sep-Pak cartridge with methanol and the solvent was then evaporated. Chromatographic analysis then proceeded by dissolving the residue from the solvent evaporation step in the chromatographic mobile phase. A Nova-Pak Ph stationary phase was used as the separation medium and detection accomplished by UV absorption. The need to evaporate the Sep-Pak elution solvent probably degrades sample recoveries and these authors suggested that the various tetracyclines could be recovered with 68–90% yields. The strong chromophore of the tetracyclines provided low detection limits, however, coefficients of variation for between-run analyses were high with values in the range 2–8%.

Similar results were reported by Horie *et al.* [42] in developing a method for the analysis of nalidizic acid, oxolinic acid and piromidic acid residues in fish. These synthetic antibiotic materials are used frequently in aquaculture, and residues of these materials have been reported in commercial fish tissues. Tissue samples were extracted/deproteinized under centrifugation with a methanol–phosphate

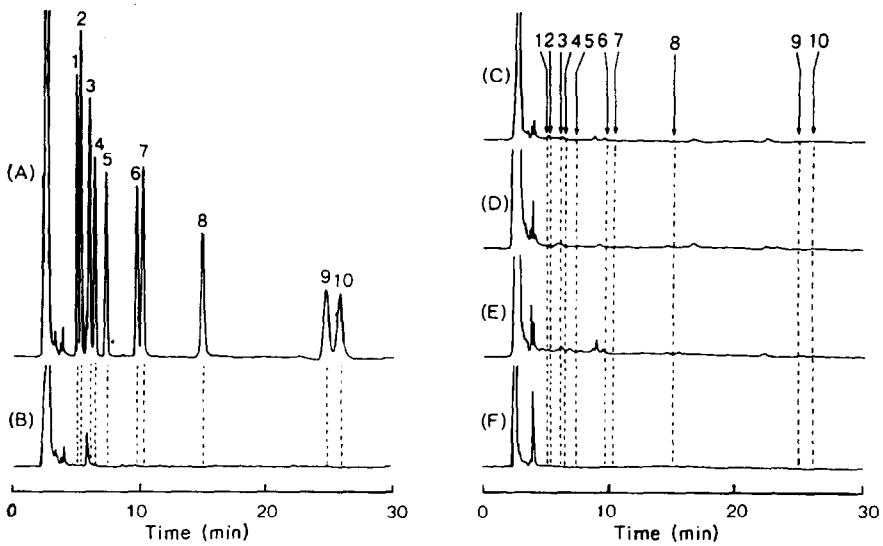

Fig. 7. HPLC separation of sulfonamide antibiotics obtained from commercially available meats, eel and egg. (A) Fortified chicken (0.5 μg/g); (B) chicken; (C) pork; (D) beef; (E) eel; (F) egg. Peaks: 1 = STZ (sulphathiazole); 2 = SDZ (sulphadizaine); 3 = SMR (sulphamerazine); 4 = SDD (sulphadimidine); 5 = SMPD (sulphamethoxypyridazine); 6 = SMMX (sulphamonomethoxine); 7 = SIZ (sulphisozole); 8 = SMX (sulphamethoxazole); 9 = SDMX (sulphadimethoxine); 10 = SQ (sulphaquinoxaline) (50 ng each). Column, Wakosil 5 C_{18} (5 μm) (250 × 4.6 mm I.D.); mobile phase, acetonitrile–0.02 *M* aqueous phosphoric acid solution (24:76, v/v); flow-rate, 1.0 ml/min; detection, 272 nm. From ref. 40.

buffer solvent. The extracted antibiotics were further isolated by Sep-Pak clean-up, eluted with methanol, the solvent was evaporated and the residues were then dissolved in the chromatographic mobile phase. A reversed-phase mode was used to separate the antibiotic compounds with either fluorescence or absorption detection of the separated materials. Excellent recoveries were reported for all three materials (> 85%), with detection limits at the 10-ng/g level.

4.2. Milk and infant formula

Milk and milk products provide a measurement matrix with many potentially interfering substances for antibiotic analysis. Analytical methodologies usually contain clean-up procedures for both protein and lipophillic substances. In an effort to overcome these problems by the use of a high-efficiency separation system, a procedure has been developed based on capillary column GC for the analysis of β-lactam antibiotics (penicillins) [43]. Even with this high-efficiency separation system, extensive sample clean-up is required. The penicillins are rendered sufficiently volatile for GC analysis by methylation with diazomethane. Excellent LODs were demonstrated (< 1 ng/g), however, the need to derivatize the antibiotic limits the application of this technique in rapid-screening applications. The extensive clean-up prior to derivatization did not reduce the amount of sample manipulation required and therefore no reduction in sample preparation time was realized.

Most reported analytical procedures for antibiotic substances in milk or milk products utilize RP-HPLC as the separation mode. Long *et al.* [44] mixed C_{18} derivatized silica with the milk sample and then prepared a column from this matrix in the barrel of a syringe (10 ml). Lipophillic substances were removed from the column by washing with hexane, and a series of eight different sulfonamides were then eluted with methylene chloride. RP-HPLC with UV detection (270 nm) was then used to separate the different sulfonamide analogues. The authors found this procedure to be sensitive, free of interferences and it provided excellent recoveries of the eight sulfonamides studied.

Several recent reports have appeared which de-

scribe methodology for the separation and analysis of penicillin antibiotics in milk. These procedures utilize an acetonitrile extraction of the milk sample. Acetonitrile precipitates much of the milk protein and the precipitated protein is subsequently removed by centrifugation. Reversed-phase chromatography is used to separate the various penicillin derivatives. Junns *et al.* [45], used an initial enzymatic hydrolysis of the milk sample to enhance discrimination against potentially interfering materials by forming the penicilloate product from the β-lactam moiety in the corresponding penicillin. The terminal aldehyde species is subsequently formed using mercury(II) chloride and the penicilloaldehyde is then extracted into methylene chloride. RP-HPLC is used to separate the different penicillin analogues and detection is enhanced by derivatization with dansylhydrazine to render the penicilloaldehyde fluorescent. The major problem with this approach is the hazardous nature of the mercury salt and the difficulty in disposing of the waste from the procedure. Moats [46] used a similar acetonitrile precipitation/centrifugation step to remove protein, followed by a methylene chloride–hexane extraction to remove lipid species. The authors chromatographed the sample at pH 7 and isolated a narrow elution fraction which was then chromatographed at pH 1.96. At this pH, penicillin G provided a narrow peak which was free from interferences. The approach will work for other penicillin analogues, however, due to their different functionalities, optimum chromatographic conditions for each analogue will have to be individually determined.

A similar procedure has also been reported by Thomas [47] for the analysis of tetracycline antibiotics in milk. The milk sample is diluted with an EDTA–phosphate buffer and the antibiotic then isolated using centrifugation. RP-HPLC with a gradient of decreasing polarity (oxalic acid to methanol) is used for concentration and separation of the tetracyclines. The procedure is rapid and provides excellent detection limits which makes this procedure very advantageous for large-scale screening. Long *et al.* [48] use a solid-phase clean-up routine for tetracycline analysis in milk samples. The authors mixed the milk sample with C_{18} modified silica, packed the mixture into a column and then washed off lipid species with hexane. The tetracyclines were then eluted with an ethyl acetate–acetonitrile (1:3) mixture and chromatographed by RP-HPLC using PDA detection (365 nm). The success with which this procedure can isolate tetracycline species without interference compensates for the time required for column preparation. An alternative procedure has been described [49] which uses a series of liquid extractions to isolate tetracyclines. The milk sample is first acidified to pH 2.7 and extracted with acetonitrile. The supernatant liquid is then adjusted to pH 8.2 and the tetracyclines are extracted into methylene chloride as an ion pair with Bu_4NHSO_4. The ion pair is disrupted by extraction of the methylene chloride with acid, and the tetracyclines are separated by RP-HPLC. In general, this procedure did not provide any advantages over the others reported in terms of reduction in sample preparation or in improvement in analytical capability.

Long *et al.* [50] have also described a solid matrix isolation procedure for chloramphenicol in which C_{18} modified silica is used to isolate this antibiotic material in milk. Although minimum sample preparation is required, the analytical capabilities of the method, particularly in light of the low-level monitoring necessary for this antibiotic, are not as good as those reported for the GC methods described earlier [4].

Shaikh and Jackson [51] defatted milk samples prior to analysis for neomycin residues by centrifugation at 4°C. TFA is then added to the supernatant to precipitate proteins which are then removed by another centrifugation step. The neomycin isolated from this step is then determined by direct injection of the supernatant liquid into an RP-HPLC system. The separation mode uses ion-pair formation with TFA, followed by fluorimetric detection of the *o*-phthalaldehyde derivative (post-column derivatization) of neomycin. Excellent LODs and recoveries from spiked milk samples were demonstrated with this method.

4.3. Eggs

The analysis of antibiotic residues in eggs requires the same considerations in terms of sample deproteinization and defatting as milk. Therefore, the procedures described earlier for milk analysis can be used, with minor modification for analysis of these same antibiotic materials in eggs. A novel pro-

cedure involving dialysis isolation and on-column concentration has been developed by Aerts *et al.* [52] for the analysis of sulfonamides in egg samples. Egg samples are homogenized with a small amount of sodium azide to enhance the water solubility of the antibiotic. The aqueous sample is then placed in a continuous-flow system where it is dialysed on-line through a cellulose acetate membrane. The small antibiotic molecules pass through the membrane and are then concentrated on a short column packed with polymeric XAD-4. The concentrating column is backflushed into the analytical column where the sulfonamides are separated by RP-HPLC (C_8). Fig. 8 shows the separation of a variety of different sulfonamide analogues using this automated sample preparation, concentration and separation system. The authors found that derivatization of the antibiotic materials wth *p*-dimethylaminobenzaldehyde (post-column) enhanced their UV detection at 450 nm by a factor of approximately 2 over their inherent absorptivity at 280 nm. In Fig. 8, the level of the various sulfonamides injected was 50 ng/g and, as is evident from the data, this method provides excellent separation and detection at this level and below.

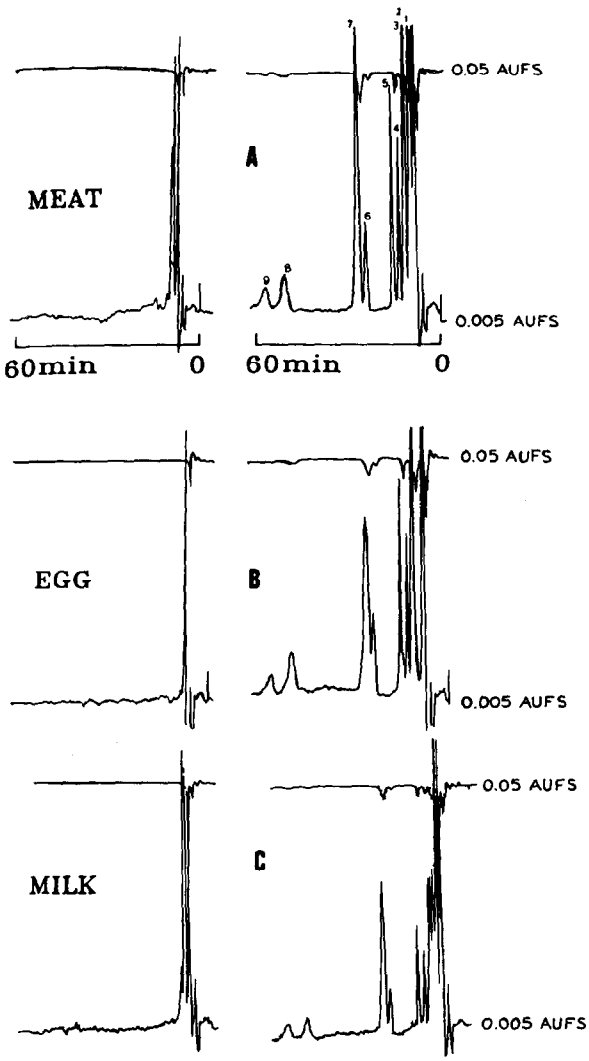

Fig. 8. Continuous-flow LC analysis of blank and spiked meat (A, 100 μg/kg), egg (B, 50 μg/kg) and milk (C, 25 μg/l) samples. Conditions: enrichment column, 60 mm × 4.6 mm I.D., XAD-4; eluent, 0.05 *M* sodium acetate (pH 4.6)–acetonitrile (82.5:17.5); analytical column, LiChrosorb RP-8; derivatization with dimethylaminobenzaldehyde (DMAB); detection at 450 nm, 0.005 a.u.f.s. Peaks: 1 = SA (sulphanilamide); 2 = STH (sulphathiazole); 3 = SD (sulphadiazine); 4 = SM (sulphamerazine); 5 = SMZ (sulphamethazine); 6 = SDX (sulphadoxine); 7 = DDS (dapsone) + STX (sulphatroxazole) + SMX (sulphamethoxazole); 8 = SDM (sulphadimethoxine); 9 = SQX (sulphaquinoxaline). From ref. 52.

With respect specifically to egg samples, this method provides significant advantages over others in that problems with emulsification are minimal. Fig. 9 shows the separation of two different sulfonamides separated from eggs using this automated method. The excellent signal-to-noise ratio obtained for sulfaguanidine suggests that this method should be able to detect several of the sulfonamides at the low ng/g level, or below. In general, one of the main advantages of this method lies in its potential for wide applicability. The realm of sulfonamides investigated with this method spanned the range from polar to relatively non-polar, and from weakly acidic to basic. This range of chemical functionalities and characteristics also encompasses many other antibiotic materials of physiological concern and the method should therefore be extendable to the analysis of these antibiotic materials in similar matrices.

4.4. Honey

As a sample matrix, honey does not present as many challenges as, for example, milk or eggs. Diaz *et al.* [53] have developed a procedure for the analysis of tetracyclines and sulfathiazole which involves simple dissolution of the honey sample in a mixture of acetonitrile–water (10:90) prior to analysis. The

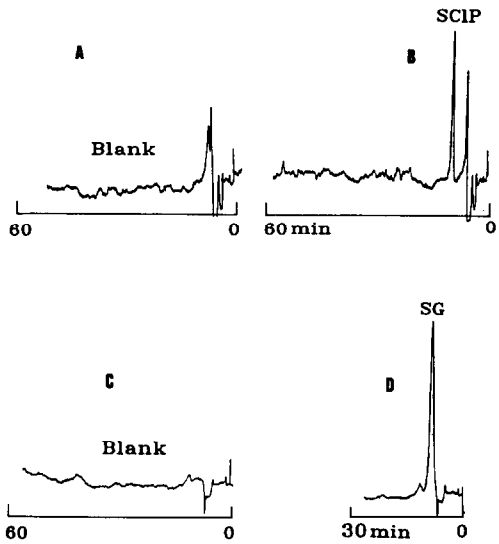

Fig. 9. Continuous-flow LC analysis of egg samples spiked with SCP (sulphachlorpyrazine) (100 μg/kg, A and B) and SG (sulphaguanidine) (50 μg/kg, C and D). Conditions for (A) and (B): enrichment column, 60 mm × 4.6 mm I.D., XAD-4; eluent, 0.05 *M* sodium acetate (pH 6.85)– acetonitrile (87.5:12.5, v/v); analytical column, C_p TM-Spher C_{18}; derivatization with DMAB; detection at 450 nm, 0.005 a.u.f.s. Conditions for (C) and (D) are the same as (A) and (B) except the eluent is water–acetonitrile–acetic acid (97:2:1, v/v/v). From ref. 52.

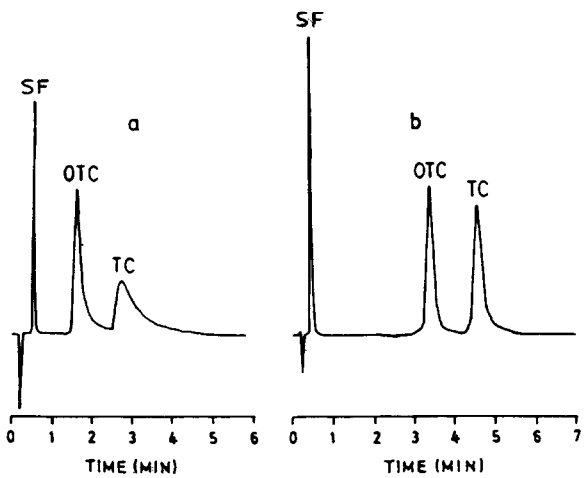

Fig. 10. (a) HPLC separation of a standard sample containing 164 ng of SF (sulfathiazole), 668 ng of OTC (oxytetracycline) and 578 ng of TC (tetracycline); mobile phase 0.01 *M* oxalic acid–acetonitrile (85:15). (b) HPLC separation of a standard sample containing 156 ng of SF, 535 ng of OTC and 622 ng of TC; mobile phase 0.01 *M* oxalic acid, 0.01 *M* SDA (sodium dodecyl hydrogensulfate)–acetonitrile (70:30). From ref. 53.

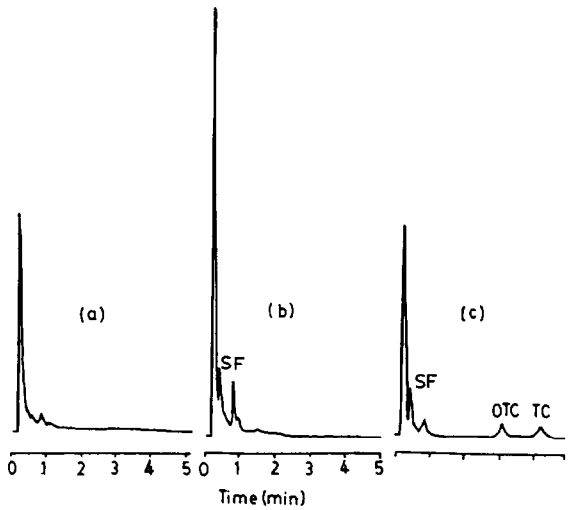

Fig. 11. HPLC separation obtained from (a) honey sample with no appreciable amount of analytes, (b) honey sample with appreciable amount of SF and (c) honey sample fortified with 10.4 μg/g SF and 23.1 μg/g OTC and TC. Chromatographic conditions are the same as in Fig. 10 (b). From ref. 53.

dissolved sample is filtered by a syringe filter and the filtrate then injected directly into the HPLC system. Micellar reversed-phase chromatography is used to separate the antibiotic species with dodecyl hydrogensulfate as the mobile phase additive. Sensitive detection was accomplished photometrically at 285 nm.

Fig. 10 clearly demonstrates the advantages of the micellar mode. In (a), severe peak tailing is evident for the two tetracycline species. In (b), an anionic micellar mobile phase was used which provided a significant reduction in peak tailing. Fig. 11 shows the application of this optimized separation system to the analysis of a honey sample spiked with these three antibiotics. The chromatographic system provides excellent separation with only minimal sample preparation. However, the detection mode utilized by these authors is not as sensitive as others reported for these antibiotics and significant improvements could be realized by application of these other modes to the analysis of tetracyclines.

5. CONCLUSIONS

As has been discussed, the monitoring of food materials for antibiotic residues is an area of in-

creasing concern and importance due to the potential impact on human health. Large-scale screening applications require methods that are rapid, accurate, provide low detection limits and are free from interference. The problem is further complicated by the wide range of chemical functionalities and modes of operation exhibited by the antibiotic materials of physiological significance in use today. As demonstrated, chromatographic methods provide many of the advantages necessary for screening applications. Judicious choice of sample preparation method, separation mode and detection strategy can provide significant immunity from problems associated with the food matrix.

Among the methods surveyed, RP-HPLC is used extensively for the analysis of many antibiotic systems as it combines relatively high separation efficiencies with low detection limits. Among the different detection modes utilized for antibiotic analysis, polarimetric detection has the potential to provide extremely selective detection of most antibiotic materials, and this selective response can minimize many of the constraints placed upon the separation system by the sample matrix. Although many of the separation modes used for antibiotic analysis are standard, separations based on capillary electrophoretic methods have much potential in the field of antibiotic analysis. Future investigations are needed to extend the generality of these techniques and expand their use into the field of food analysis.

6. ABBREVIATIONS

CE	Capillary electrophoresis
CMC	Critical micelle concentration
DMF	Dimethylformaldehyde
GC	Gas chromatography
HFBA	Hexafluorobutylacetate
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
LC	Liquid chromatography
LOD	Limit of detection
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
PDA	Photodiode array
RP-HPLC	Reversed-phase high-performance liquid chromatography
RP-TLC	Reversed-phase thin-layer chromatography
SDS	Sodium dodecyl sulfate
SFC	Supercritical fluid chromatography
TAA	Tetraalkylammonium
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TMS	Trimethylsilane
TSP-MS	Thermospray mass spectrometry
UV	Ultraviolet

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