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REVIEW

BIOANALYTICAL APPLICATIONS OF SUPERCRITICAL FLUID CHROMATOGRAPHY

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CONTENTS

LIST OF ABBREVIATIONS

CI	Chemical ionization
DFI	Direct fluid introduction
EI	Electron-impact ionization
FID	Flame ionization detection
FTIR	Fourier transform infrared detection
GC	Gas chromatography
(HP)LC	(High-performance) liquid (column) chromatography
I D	Internal diameter
MS	Mass spectrometry
PSS	Phase-system switching

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SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
THC	Tetrahydrocannabinol
UV–VIS	Ultraviolet–visible (absorption detection)

1 INTRODUCTION

Although supercritical fluid chromatography (SFC) was introduced in the early 1960s [1–3], it was not until the early 1980s that the main breakthrough of the technique took place This delay was mainly caused by the technological difficulties in developing the proper instrumentation The evolution of high-pressure technology in, for example, high-performance liquid chromatography (HPLC) and the advances in capillary-column technology in gas chromatography (GC) led to the recent revival of SFC, which has now become a valuable tool in analytical chemistry The technique is reviewed in several excellent papers [4–7]. A book covering most aspects of SFC has appeared, edited by Smith [8], and a comprehensive bibliography is available from White Ass [9], both as a book and a computer-searchable database A compilation of SFC applications has been made by Markides and Lee [10] Nowadays, SFC contributes significantly in several application areas, e g in separation of high-boiling petrochemicals [11,12] and of relatively non-polar polymers [13,14] Areas in which SFC has yet to prove itself include bioanalysis

This paper is meant to be an introduction to SFC from bioanalytical perspectives Possible applications are reviewed, and the prospects are (super)critically discussed Special attention will also be given to the various detection techniques applicable in SFC

The reference list at the end of this paper is not claimed to be complete, although most biomedical applications are mentioned there The authors are aware of the fact that this discussion may be obsolete quite soon, owing to the very rapid developments in SFC Most of the papers on biomedical applications of SFC, which are referred to in this review, have been published in the past two years

2 SUPERCRITICAL FLUID CHROMATOGRAPHY

Supercritical fluid chromatography is a column chromatographic technique, in which a supercritical fluid is used as the mobile phase A supercritical fluid is a gas or a liquid brought to a temperature and a pressure above the critical point Critical parameters of several compounds, which may be considered for use as mobile phases in SFC, are given in Table 1 Carbon dioxide is applied as the mobile phase in most cases, because its critical parameters, i e a critical temperature of 31 °C and a critical pressure of 7 3 MPa, are rather favourable and, moreover, because it is cheap, non-toxic and non-inflammable

TABLE 1

CRITICAL PARAMETERS OF POSSIBLE SUPERCRITICAL FLUID CHROMATOGRAPHY SOLVENTS

Compound	Critical temperature (°C)	Critical pressure (MPa)	Critical density (g/ml)	Solubility parameter [4] ($cal^{-1/2}\ cm^{-3/2}$)
Pentane	197	3 3	0 23	5 1
Diethyl ether	194	3 6	0 27	5 4
Sulphur hexafluoride	45	3 7	0 74	5 5
Ethane	32	4 8	0 20	5 8
Acetonitrile	275	4 8	0 25	6 3
Nitrous oxide	36	7 2	0 45	7 2
2-Propanol	235	4 7	0 27	7 4
Carbon dioxide	31	7 3	0 45	7 5
Methanol	241	7 9	0 28	8 9
Ammonia	132	11 3	0 24	9 3
Water	374	21 8	0 32	13 5

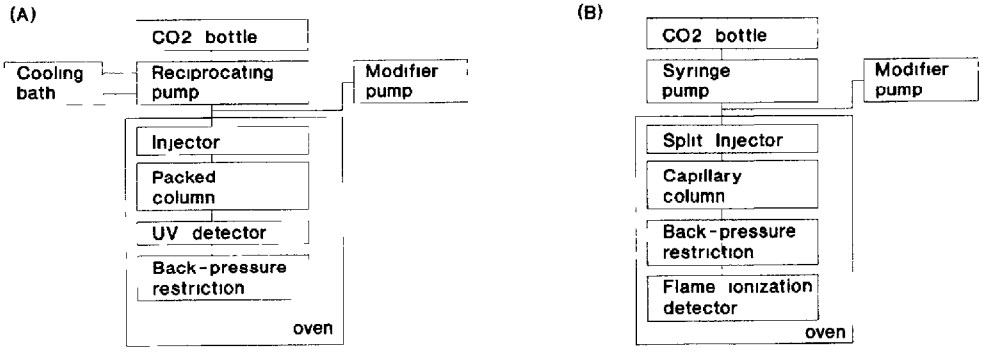

Fig 1 Block diagram of SFC instrumentation for (A) packed columns and (B) capillary columns

A block diagram of basic SFC instrumentation is given in Fig 1. The mobile phase is delivered by a high-pressure pump, which may either be a reciprocating piston pump or a syringe-type displacement pump The sample is usually injected as a solution by means of a high-pressure injection valve The column may either be a packed column, comparable with HPLC columns with respect to dimensions and packing materials, or an open capillary column, comparable with capillary GC columns but with somewhat smaller internal diameters (50–100 μm) Detection can be performed either on-line, using for instance UV or fluorescence detectors, or after expansion of the fluid, using for instance flame ionization detection (FID) or mass spectrometry (MS) The mobile phase is kept under supercritical conditions by means of a restrictor until either on-

line detection has been performed or just before the expansion into a gas-phase detector. Various restriction types are discussed by Smith et al [6].

The retention in SFC is based on the selective interactions of the analyte with the mobile and stationary phases The retention characteristics are influenced by several parameters, of which, besides the nature of mobile and stationary phases and the type of solute, the density of the mobile phase and the temperature are the most important

The polarity of pure mobile phases for SFC can be characterized by means of the solubility parameter [15,16], which is given for several compounds in Table 1 A high solubility parameter corresponds to high polarity The solubility increases when the solubility parameters of the analyte and the solvent are more alike Carbon dioxide acts as a solvent of intermediate polarity Only a few compounds that are suitable as the mobile phase in SFC have a higher solubility parameter, e g ammonia, nitrous oxide and water, but for several reasons these solvents are not suitable alternatives to carbon dioxide ammonia and nitrous oxide for obvious reasons related to their toxicity and water because of its very high critical parameters and because of the destructive action of supercritical water towards most materials The use of ammonia and nitrogen dioxide as mobile phases in SFC has been demonstrated [17,18]. SFC with pentane or other solvents is also under investigation (see for instance the excellent reviews by Klesper and Schmitz [7,19], but will not be discussed here, since those fluids find little application in the bioanalytical field

Once an appropriate compound has been selected as the mobile phase (carbon dioxide in most cases), the solubilizing properties of the supercritical fluid

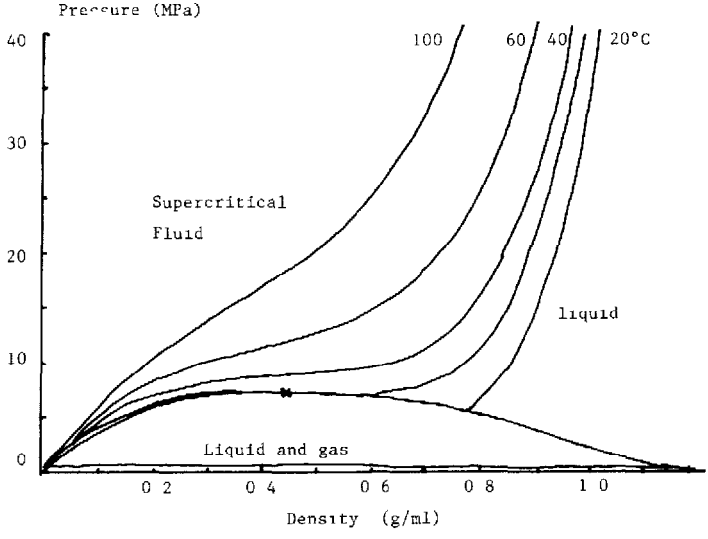

Fig 2 Phase diagram (density versus pressure) of carbon dioxide

can be influenced by means of the density The density of the fluid can be controlled by means of the temperature and the pressure, according to the phase diagram given in Fig 2. By variation of temperature and pressure the density of pure carbon dioxide as a supercritical fluid can be varied between 0 2 and 0 9 g/ml, which in practice enables the chromatographic elution of many relatively non-polar compounds In capillary SFC with non-polar stationary phases the density can be considered as a parameter comparable with the temperature in capillary GC By applying density programming, relatively low-polar compounds in a wide range of molecular masses can be separated, such as dimethylpolysiloxanes (Fig 3) [13] It has been argued that working at higher pressures is more practical since the density of the fluid is less influenced by fluctuations in the pressure

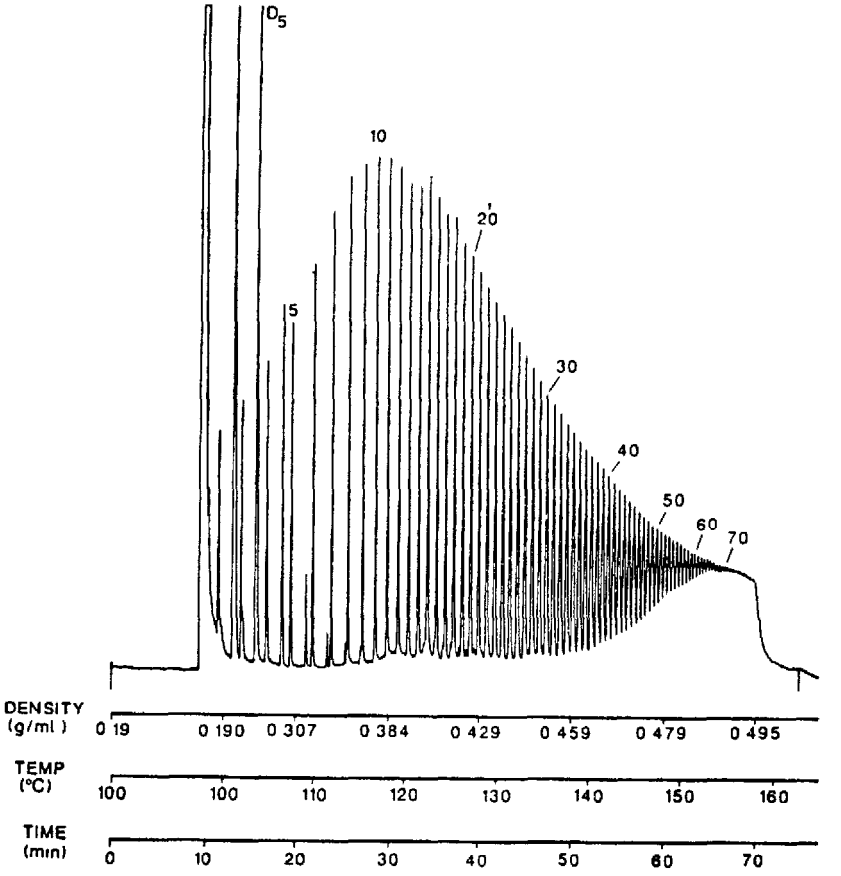

Fig 3 Example of the application of density- and temperature-programmed capillary SFC in separation of polymeric compounds Chromatogram of dimethylpolysiloxane (Reproduced from ref 13 with permission)

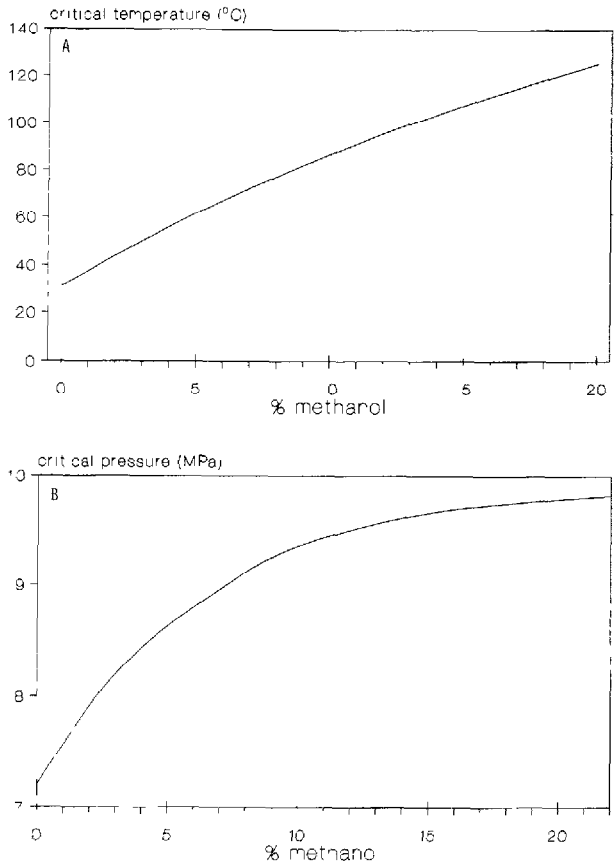

Fig 4 Critical parameters [(A) temperature and (B) pressure] for carbon dioxide–methanol mixtures, calculated according to ref 27

In many bioanalytical applications high densities are needed because of the polarities of the compounds involved When the chromatographic elution of the more polar compounds cannot be affected satisfactorily by increasing the density, the polarity and thus the solubilizing characteristics of the mobile phase can further be influenced by the addition of polar modifiers, such as methanol The action of the modifiers in SFC is not completely understood The coverage of active sites on the stationary phase materials, e g residual silanol groups, is generally considered as being one of the important features of the addition of modifiers [20,21], but since high percentages of modifiers (up to 15%) are used in some bioanalytical applications, the influence on the polarity of the mobile phase might be important as well. Solute–solvent interactions have been investigated, for instance using the solvatochromic technique [22,23] The selection of the modifier has been discussed in various pa-

TABLE 2

COMPARISON OF PACKED COLUMN AND CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY

Feature	Packed column (LC-like)	Capillary column (GC-like)
Column length	50–250 mm	1– 20 m
Inner diameter	1– 4 6 mm	50–100 μm
Stationary phase	5– 10 μm particles	0 25 μm film
Separation speed	High	Low
Efficiency	Moderate	High
Sample loadability	High	Low
Mobile phase density	High	Gradient
Detector	LC-type UV	GC-type FID
Applications	SFC–MS	Analytes not amenable to capillary GC
	Simple samples	Complex samples
	Target compound analysis	Fingerprinting

pers [24,25] Modifiers are especially used in packed column SFC, although the use of modifiers in capillary SFC also receives considerable attention nowadays [26]

It is important to notice that addition of a modifier changes the critical parameters of the mobile phase Critical parameters of several solvent mixtures calculated using equations given by Chueh and Prausnitz [27] have been reported [26,28] Methanol is used as the modifier in most cases Fig 4 gives a plot of the critical parameters (pressure and temperature) of methanol–carbon dioxide mixtures It must be pointed out that several applications in which modifiers are used are actually applications of subcritical fluid chromatography, because the separation is performed at temperatures below the critical temperature. From a practical point of view the difference between subcritical and supercritical conditions with respect to temperature appears not to be very important

SFC is performed either with open tubular columns (capillary SFC) or with packed columns Extensive comparison of capillary and packed columns for SFC from both theoretical and practical points of view is given in several papers [4–6, 29–31], and the most important differences are listed in Table 2 In general, it can be stated that packed column SFC is more like LC, whereas capillary SFC shows more of the characteristics of capillary GC A more detailed discussion will be given below

In capillary SFC, fused-silica columns are used with immobilized, cross-linked polysiloxane stationary phases, showing non-polar, polarizable characteristics, depending on the type of polysiloxane used (e g dimethyl-, phenyl-, cyanopolysiloxanes, and mixed phases) In packed column SFC conventional LC packing materials are used in most cases, e g. silica and chemically modified silica materials, such as octadecyl-, aminopropyl- and cyanopropylsilica The

use of chiral stationary phases for the SFC separation of enantiomers has also been described for both capillary [32,33] and packed column SFC [34,35]

3 SFC IN BIOANALYSIS

Bioanalysis is the qualitative and/or quantitative analysis of biologically active compounds, for instance drugs and their metabolites, pesticides, and also endogenous compounds, in biological matrices. A bioanalytical method is a combination of four related steps: sample pretreatment, separation, detection and data handling. Most attention is given in this paper to two of these steps: separation and detection.

With respect to the bioanalytical applications of SFC several aspects are of importance In most cases (very) polar neutral or ionic compounds have to be analysed, which is impossible with pure carbon dioxide as the mobile phase Therefore, frequent use is made of various modifiers in the mobile phase The injection volume is limited in capillary SFC (typical injection volume 1–10 nl) by the column dimensions and the sample loadability of the stationary phase, and in packed column SFC, where the injection volume is typically 1–5 μl of liquid with 4 6 mm I.D. columns Larger injection volumes in packed column SFC result in pressure instabilities in the system, probable caused by disturbance of the supercritical conditions of the mobile phase. In general, large injection volumes (20–100 μl) are preferred where possible in most bioanalytical applications, because it is desirable to inject the equivalent of, for instance, 1 ml of plasma after sample pretreatment directly on to the column for reasons of detectability. Sample pretreatment procedures resulting in ca 10–20 μl of the sample solution are difficult to develop and to perform reproducibly The choice of the detection system is of course also important This aspect will be discussed in more detail below

Only a few examples of (semi-)quantitative bioanalysis by SFC have been reported Many of the papers referred to in the next two sections indicate the possibility of analysing particular compounds with SFC but do not describe the analysis of those compounds in biological matrices demanding a sample pretreatment. Since in most papers concentration levels of the compounds analysed are omitted, no deductions can be made about the quantitative aspects Although some work has been reported on combined SFC–Fourier transform infrared spectrometry (FTIR), from which qualitative identification of (unknown) compounds can be obtained [36,37], the most convincing results in qualitative bioanalysis are obtained with SFC–MS [6] In the following two sections the results obtained from the application of SFC in bioanalysis and in the analysis of biomedically interesting compounds will be reviewed for capillary and packed columns, respectively

4 APPLICATIONS OF CAPILLARY SFC

Capillary columns are especially useful in SFC because they offer highly efficient separation, which is necessary in the analysis of complex samples, easy density programming, and the use of GC-type detectors, especially FID Compounds can be analysed with capillary SFC that are not sufficiently volatile or stable for GC analysis, and in comparison with LC very high column efficiencies are attainable The most important disadvantage of capillary columns in (quantitative) bioanalysis is the low sample loadability, which is inherent to capillary columns Typically, relatively short columns (1–15 m) are used, with 50–100 μm I D , on to which 1–10 nl of sample are injected (after splitting)

Capillary SFC has found extensive use in various areas, and some of the biomedically interesting applications are summarized in Table 3 A wide range of compounds has been analysed successfully From these applications some examples have been selected for a somewhat more detailed discussion

The analysis of various classes of drugs by means of capillary SFC has been described by Later et al [38] A chemically bonded methylpolysiloxane film (SE-33) in 13–18 m$\times$50 μm I D columns is used as the stationary phase and carbon dioxide without a modifier as the mobile phase Chromatograms are given for oxytetracycline, corticosteroids and cannabinols, the latter two not only for standard solutions, but also for equine and human urine extracts The clearly distinguishable peak of prednisolone from an equine urine extract, not present in the blank chromatogram, is a nice demonstration of the real-world applicability of SFC The separation of tetrahydrocannabinol (THC) and six

TABLE 3

BIOMEDICAL APPLICATIONS OF CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY

Category of compounds	References
Alkaloids	71
Amino acid derivatives	32, 33
Antibiotics, tetracyclins	18, 38, 40, 72
Cyclosporin	40, 72
Drugs and drugs of abuse	17, 38, 73
Fatty acids and glycerides	39, 74, 75
Oligosaccharides	76
Pesticides, herbicides and insecticides	77, 78, 79
Prostaglandins	74
Steroids	38, 73, 80
Trichothecene mycotoxins	81
Uracils and uridines	82
Vitamins	40

of its metabolites enables the quantitation of these compounds The peak obtained from a sample containing ca 400 ng/ml of 9-carboxy-THC gives an excellent signal-to-noise ratio For several other compounds, such as erythromycin and drugs of abuse (e g cocaine, methadone, phenobarbital), chromatographic conditions are indicated in the original paper [38]. Capillary SFC in combination with FID, as described in that paper, is especially useful for the analysis of polar drugs, which do not contain (strong) chromophoric groups

FID in capillary SFC was also used by Hawthorne and Miller [39] in the analysis of commercial waxes which, depending on their nature, may contain a wide variety of compounds without chromophoric properties such as saturated hydrocarbons, long-chain carboxylic acids and their esters, and mono-, di- and triglycerides Separation of the various groups of compounds and the various compounds within a group can be achieved with SFC An example of this is given in Fig 5 Identification and molecular mass determination of the various compounds in these samples is performed by on-line coupling with MS

Capillary SFC of cyclosporin, two closely related ionic polyether antibiotics and some fat-soluble vitamins was demonstrated by White et al [40] The reproducibility of the analytical system with respect to retention time and peak area was tested with a 660-ppm provitamin D solution The 2 m $\times$ 250 μm I D DB-WAX column was operated isothermally at 130°C with linear pressure programming The measured values (mean $\pm$ S D , $n=8$) of the retention time

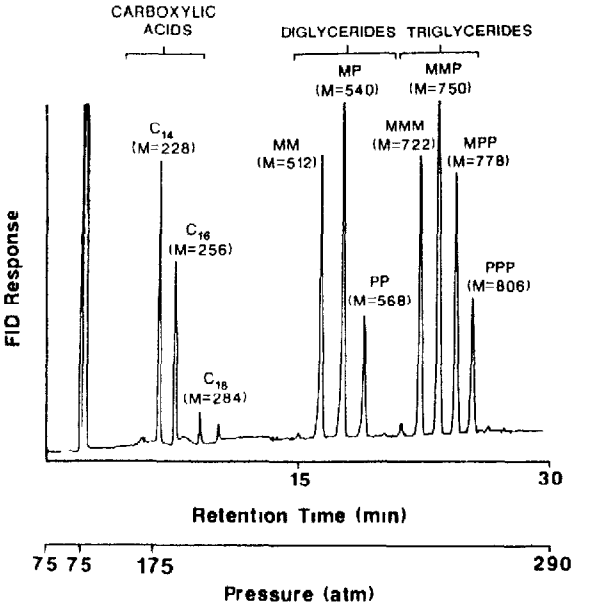

Fig 5 Capillary SFC–FID chromatogram demonstrating the separation of various groups of compounds and the various compounds within a group (Reproduced from ref 39 with permission)

and the peak area were 22.47 ± 0 022 min and (1 22 ± 0 021) · 10^6 counts, demonstrating an excellent run-to-run reproducibility.

The last example discussed here is the capillary SFC analysis of the isopropyl ester -3,5-dinitrobenzamide derivatives of racemic mixtures of amino acids on a 5 m × 50 μm I D fused-silica column coated with an N-naphthylamino acid ester-derivatized polymethylsiloxane phase (Pirkle type) [32] The enantioselectivities (defined as the ratio of capacity ratios) obtained in these preliminary experiments are different from and lower than those obtained in packed column LC using a comparable stationary phase [32] In our opinion the separation of enantiomers on capillary columns with SFC is of great interest The high efficiencies that can be achieved with capillary columns may compensate for the lower enantioselectivities Various other chiral stationary phases for capillary SFC have recently been described [33] Significant progress is expected in this field.

In summary, it can be stated that several interesting biomedical applications of capillary SFC have been demonstrated In combination with either FID or MS, capillary SFC is a powerful tool in the analysis of complex mixtures

As with all capillary column techniques the sample loadability is limited. Recent advances in sample loadability in capillary GC by the use of thick-film columns demonstrate that these loadability limitations can be diminished to some extent The development of special stationary phases for SFC is still in its infancy. The use of supercritical mobile phases modified with polar additives will probably further extend the range of application of capillary SFC.

5 APPLICATIONS OF PACKED COLUMN SFC

Packed columns are especially useful in SFC because they combine moderate efficiencies with short analysis times, they have high sample loadability, are easy to use with and without modifiers and allow the use of LC-type detectors, such as UV and fluorescence. The most important disadvantages of packed columns are the presence of active sites on the stationary phase material, which may lead to excessive tailing with polar compounds, and the relatively high pressure drop over the column, resulting in a gradient of the solubilizing characteristics of the mobile phase over the column length. Typically, conventional columns are used (100–250 mm × 1–4 6 mm I D) packed with common LC materials Injection volumes are 1–5 μl The analytes are frequently dissolved in methanol or dichloromethane In some recent papers, deactivated stationary materials have been described [41], and the use of packed fused-silica columns for SFC has also been demonstrated [42] Although the applicability of various modifiers has been demonstrated, e g by Levy and Ritchey [25], methanol is used most often

The biomedically interesting applications of packed column SFC are sum-

marized in Table 4. Some of these applications will be discussed in more detail below

A wide variety of biomedically interesting compounds has been investigated by Berry and co-workers [43,44] using combined packed column SFC and MS Data are reported on the analysis of xanthines, carbamates, sulphonamides, steroids, ergot and indole alkaloids, coumarins, antibiotics and veterinary drugs [43,44], not only from standard solutions but also from plant extracts. It is striking that various impressive results in packed column SFC are in fact obtained by using SFC–MS. Lane et al [45], for instance, demonstrate the analysis of cephalosporin esters, avermectins and erythromycin A An example of a packed column SFC separation of erythromycin and 2′-acetylerythromycin, as obtained in the authors' laboratory, is given in Fig 6 By decreasing the percentage of modifier separation can also be obtained of 2′-acetylerythromycin and 2′-ethylsuccinylerythromycin, which coelute under the conditions used in Fig 6, but then erythromycin itself has a very high capacity ratio and gives a broad peak

Enantiomeric separation by packed column SFC has also attracted considerable attention. Mourier et al [34] were the first to demonstrate the separation of enantiomeric compounds on a Pirkle-type stationary phase The use of an (N-formyl-L-valylamino)propylsilica stationary phase [35] and a β-cyclodextrin-bonded stationary phase [46] has also been described Another approach to chiral separation, i e the use of chiral mobile phase additives, has been demonstrated for SFC by Steuer et al [47] Enantiomeric 1,2-diaminoalcohols of pharmacological interest, such as pindolol, metoprolol, oxprenolol and propanolol, have been separated on a cyano-bonded phase with 20%

TABLE 4

BIOMEDICAL APPLICATIONS OF PACKED COLUMN SUPERCRITICAL FLUID CHROMATOGRAPHY

Category of compounds	References
Alkaloids, (opium, ergot, indole)	43, 44, 56
Amino acid derivatives	35
Antibiotics	18, 43, 44, 45, 49
Avermectins	45
Cephalosporin esters	45
Coumarins and coumarin rodenticides	44
Drugs and drugs of abuse	24, 28, 83
Pesticides, herbicides, insecticides	43, 44, 50, 52, 53, 83, 84, 85
Saccharides	57
Steroids (ecdy, other)	24, 43, 86
Triglycerides	57, 86

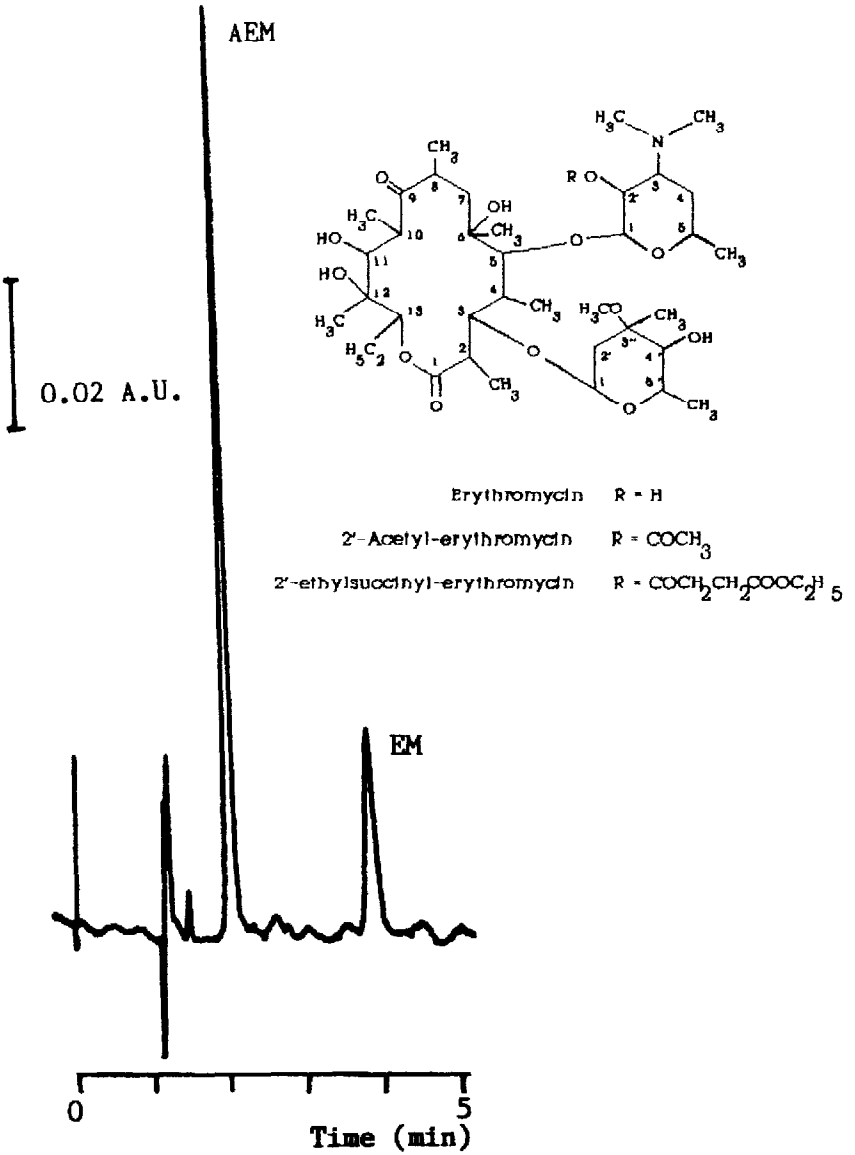

Fig 6 Separation of 2′-acetylerythromycin (AEM) and erythromycin (EM) by means of packed column SFC UV (210 nm) Conditions 150 mm×4 6 mm I D column packed with 5-μm aminopropyl silica, 2 ml/min 15% methanol in carbon dioxide at 30 MPa, oven temperature, 60°C

acetonitrile, containing two ion-pairing agents, in carbon dioxide The two ion-pairing agents are N-benzoxycarbonylglycyl-L-proline, which gives diastereoisomeric ion pairs with cationic solutes, and triethylamine, which acts as a counter ion Parameters controlling the retention and the selectivity in the

SFC separation have extensively been studied for this mobile phase system [47] The use of ion-pairing agents as a modifier in packed column (and capillary) SFC is a very interesting approach for the analysis of ionic compounds Tetramethyl ammonium hydroxide has been used as ion-pairing agent in the analysis of phenylhydantoin amino acid derivatives [48] Other applications in this direction will certainly be reported

Packed column SFC can offer moderate efficiencies in the rapid analysis of mixtures containing not too many compounds, which might for instance be useful in screening. The applicability of this feature of packed column SFC has clearly been demonstrated by Crowther and Henion [28], using detection by MS Crude equine urine extracts containing phenylbutazone and its metabolites are separated within 5 min [28]

On-line solid-phase isolation of thermally labile compounds from plasma and packed column SFC has been investigated by Niessen and co-workers [49,50], using valve-switching techniques The analytes are adsorbed form aqueous solutions, e g. plasma, onto a solid adsorbent, which after washing and drying is switched into a stream of supercritical fluid, which desorbs the analytes and transfers them to the analytical column, where they are separated using packed column SFC This approach, which actually is a special application of phase-system switching (PSS) [51], can be used as an on-line sample pretreatment method the analytes are isolated from an aqueous biological matrix, which is incompatible with SFC Significant preconcentration of the analyte takes place on the trapping column, because actually 1 ml of plasma is injected directly onto the chromatographic system For the herbicide diuron, for instance, the UV detection limit following injection of 5 μl of methanolic solutions is ca 50 ng, corresponding to a concentration of 10 μg/ml, whereas with PSS a similar amount of diuron can be detected in 1 ml of aqueous solution A preconcentration by a factor of 200 is achieved As an example of this approach the UV chromatogram of plasma spiked with 60 ng/ml diuron and injected directly onto the trapping column is given in Fig 7 When, instead of a UV detector, MS detection is performed, it becomes clear that the selectivity of PSS as a sample pretreatment method is rather limited in this case, since several other compounds coelute from the trapping column [50]

Another powerful method for analysing (biological) matrices, which also uses valve-switching techniques, is the combination of supercritical fluid extraction (SFE) and other chromatographic techniques, for instance SFC The on-line SFE and SFC analysis of sulphonylurea herbicides and their metabolites from complex matrices, such as soil, plant materials and cell cultures, has been demonstrated by McNally and Wheeler [52] Optimization of experimental parameters influencing the extraction efficiencies in these types of experiments are performed using diuron and linuron as model compounds [53] On-line SFE–SFC of plant material has also been demonstrated by Engelhardt and Gross [54] On-line SFE–SFC–MS and SFE–SFC–MS–MS of veterinary

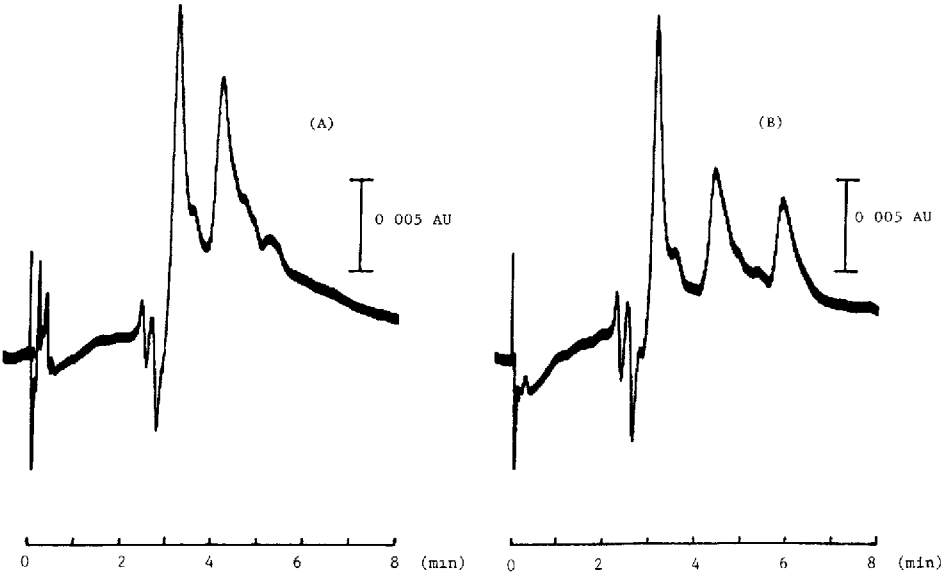

Fig 7 Chromatograms of (A) blank plasma and (B) plasma spiked with 60 ng/ml diuron after phase-system switching Conditions C_{18} analytical column (150 mm×4 6 mm I D), C_8 trapping column (10 mm×3 2 mm I D), 2 ml/min 2% methanol in carbon dioxide, 30 MPa, oven temperature, 50 °C, UV detection at 250 nm

drugs from kidney samples have been reported by Games et al [55] The use of these on-line methods improves the overall performance of the analytical system in bioanalysis Larger samples can be automatically analysed with only minor sample pretreatment The use of on-line SFC–MS and SFC–MS–MS can afford direct identification of constituents in the complex mixtures Significant progress may be expected from this exciting field in the near future

In summary, it can be stated that several interesting biomedical applications of packed column SFC have been reported Some results on quantitative analysis of compounds in biological matrices have been described [49,50,56,57] However, most attention in packed column SFC is focused on qualitative analysis, especially using on-line SFC–MS This field of application gives a strong impetus to the developments in packed column SFC It must be pointed out that the same statement can be made to some extent for combined capillary SFC and MS, although in that case the analysis of compounds with higher molecular masses, for instance polymers, is at present more important The areas of application of packed column SFC can probably be extended further by investigating other modifiers The application of ion-pairing agents is a clear example of this The use of other stationary phases, for instance polymeric phases based on styrene–divinylbenzene copolymers, might be useful in

biomedical applications, where the analysis of polar compounds is often hampered by strongly tailing peaks

6 DETECTION TECHNIQUES IN SFC

Little attention has been paid in this review to the instrumental aspects of SFC Such a discussion is not as important now as it was some years ago, because dedicated capillary or packed column SFC instruments are now available from several manufacturers The instrumental aspects of SFC have been discussed in more detail in several papers [6,8,58–60] Back-pressure restrictions are dealt with in several papers [6,8,61–64], although the perfect restrictor has not yet been found The reproducible injection of small volumes in capillary SFC is also addressed in several papers, a review on this topic has recently been given by Andersen [65]

This section is mainly devoted to the various detection techniques in use with SFC The detectors that have been applied are listed in Table 5 References are given for the less common detectors, which are not discussed further in this paper Several typical GC detectors are used only in capillary SFC Several powerful GC detectors, e g nitrogen–phosphorus and electron capture, are used hardly at all in capillary SFC

FID is a universal and sensitive technique, which is well known from its application in GC The detector gives a quantitative response for organic compounds, so its application is more or less limited to separations in which pure carbon dioxide, or some inorganic compound, is used as the mobile phase, polar organic modifiers produce high background and consequently high noise levels

TABLE 5

DETECTORS APPLIED IN SUPERCRITICAL FLUID CHROMATOGRAPHY

Detection principle	References
Flame ionization detector	–[a]
Fluorescence detector	87
Fourier transform infrared detector	36, 37
Inductively coupled plasma atomic spectrometry	88
Ion mobility spectrometry	89
Light scattering detector	57
Mass spectrometry	–[a]
Microwave-induced plasma detector	90, 91
Nitrogen–phosphorus detector	79
Nuclear magnetic resonance detector	92
Photoionization detector	93
UV–VIS and photodiode array detectors	–[a]

[a]These detectors are discussed in the text

Examples have been given in an earlier section It is important to notice that with FID the detection takes place after decompression of the mobile phase High demands are put on the design of the back-pressure restrictor at the end of the analytical column [61,62] To some extent it can be considered as a drawback of FID that the detector does not give any information on the identity of the analytes SFC–FID is especially attractive when the analytes are not amenable to GC because of their limited volatility or thermal stability, and not amenable to LC because of the absence of (sufficiently strong) chromophoric groups

While FID can be considered as the most common detection method in capillary SFC, the UV–VIS detector is most often used in packed column SFC. Conventional LC UV detectors are used, equipped with modified flow-cells to resist the high pressures of the supercritical phase. Decompression of the mobile phase then takes place after the detector UV–VIS detectors can provide some compound-specific information, especially when a photo-diode array detector is used, as described by Jinno et al [66] When an appropriate high-pressure flow-cell is available, packed column SFC–UV is relatively straightforward In contrast to this, capillary SFC–UV imposes some additional requirements, especially with respect to flow-cell dimensions and external peak broadening [67] Capillary SFC–UV is of interest when some analyte-specific information is desirable and/or when the use of FID is ruled out because polar organic modifiers are needed for a particular application

7 COMBINED SFC-MS

The mass spectrometer is another important and frequently used detector in SFC In the sections on the applications of capillary and packed column SFC several interesting examples of combined SFC–MS have already been described At present, it appears that coupling with a mass spectrometer is an important bioanalytical application area of SFC Therefore, combined SFC–MS is discussed here in a separate section

MS is unsurpassed in its ability to detect analytes eluting from a chromatographic column both universally and selectively with low detection limits, and in its ability to give structural information on very small amounts of analyte The on-line combination of both packed column and capillary SFC with MS has received much attention, an excellent review on this topic has been written by Smith et al [6]

On-line (packed column) SFC–MS was described for the first time by Randall and Wahrhaftig [68] Since then various interface designs have been reported, which can be divided into four groups supersonic beam systems [69], direct fluid introduction (DFI) [6], moving belt interfaces [43] and thermospray [44,70] The latter three are most often used in practice These interfaces are actually modifications of interfaces used in LC–MS In DFI and

thermospray interfaces the fluid is expanded directly into the ion source, whereas with the moving belt interface the supercritical fluid is removed when the column effluent is sprayed onto the moving belt

Ionization of the analytes can be performed with either electron impact (EI) ionization or chemical ionization (CI) EI is possible with narrow capillary columns in direct introduction modes and with the moving belt interface EI is ideally suited for obtaining structural information of the analyte. CI is possible by using mobile phase constituents as the reagent gas, or with additional reagent gases when either narrow capillary columns in direct introduction modes or the moving belt interface is used When the mobile phase constituents are used as the reagent gas, either charge-exchange mass spectra are obtained, containing EI-like structural information, or molecular mass information from protonated molecules, resulting from proton transfer ion–molecule reactions with protonated molecules of the polar modifier present in the SFC mobile phase EI and charge-exchange CI are most often used in capillary SFC, and both charge-exchange and proton transfer CI are mostly used in packed column SFC

The various applications of packed column and capillary SFC have been discussed in the previous sections It is beyond the scope of this paper to elaborate further on the various MS aspects of SFC–MS

8 CONCLUSIONS AND PERSPECTIVES

The review of the various bioanalytical applications of SFC and discussion on various aspects given above shows that great progress has been made during the past few years Although it must be pointed out that hardly any quantitative work has yet been performed, the prospects are good More dedicated sample pretreatment methods, based on SFE for example, have to be developed In qualitative analysis SFC has proven its value, especially in combination with MS Application of SFC–MS has yielded some of the more impressive results in bioanalytical SFC Several aspects will probably receive more attention in the near future, e g separation of enantiomers, the use of ion-pairing agents, which opens a new and broad field of application for SFC, and the development of other stationary phase materials Hardly applied so far, but probably very useful, is chemical derivatization of analytes, especially to make typical LC analytes more amenable to (capillary) SFC by means of GC-like derivatization procedures Considerable progress in SFC can be expected in the next few years, especially considering the fact that over 50% of the references in this paper have been published in the past two years

A review paper like this should make some prediction of the impact that SFC will have on the bioanalytical practice in the near future Such a prediction is of course risky, owing to the present state of early development of bioan-

alytical applications of SFC Both capillary and packed column SFC will be introduced in some routine laboratory procedures quite soon, to solve specific problems As with LC, the introduction of SFC into laboratories where many different samples have to be analysed will take more time, although density-programmed capillary SFC might be an easier approach to separation problems than reversed-phase LC A rough estimate would be that SFC might be used instead of LC in at least 25% of the bioanalytical applications However, the conservatism of scientists and institutes will keep the number of SFC applications relatively low The question "Why use SFC, when we can do it with LC?", like the old question "Why use capillary GC, when we can do it with packed column GC?", is of course a valid one Unfortunately this slows down the developments in SFC that might yield great (bio)analytical benefit in many applications

Sensitivity, selectivity, speed and costs are important parameters with respects to the implementation of new analytical methods in the laboratory In comparison with LC, the most obvious competitor of SFC, some sensitive detectors can be used that are not well suitable for routine LC, e g FID and nitrogen–phosphorus detectors, while other powerful LC detectors, such as UV and fluorescence, can be used as well Speed can be higher with packed column SFC, and the high efficiencies attainable in capillary SFC may avoid complicated and time-consuming multidimensional LC separations LC is cheaper because the computer-controlled programmable SFC pumps are still rather expensive With respect to selectivity, perhaps the most important parameter in bioanalysis, it is not clear what the position of SFC relative to LC is or will be in the near future The development of, perhaps dedicated, sample pretreatment procedures for SFC, is a key point in the assessment of selectivity Very little work has been done in this respect Summarizing, it can be stated that SFC can play an important role, although some breakthroughs in selectivity and sample pretreatment and some eye-catching applications are probably necessary to convince most scientists of this rather optimistic view of SFC

9 SUMMARY

Supercritical fluid chromatography (SFC) has become a valuable tool in analytical chemistry In the past few years considerable progress has been made in bioanalytical applications of both capillary and packed column SFC Progress in this respect has especially been made with combined SFC–mass spectrometry, an important alternative to combined liquid chromatography–mass spectrometry The various applications of capillary and packed column SFC are reviewed, while special attention is given to detection technique applicable in (bioanalytical) SFC Considerable progress in SFC is expected in the near future

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