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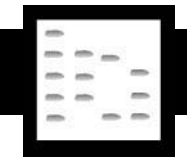
STEROLS



Supercritical Fluid Chromatography

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Introduction

In supercritical fluid chromatography (SFC) the mobile phase is a fluid subjected to pressures and temperatures near or above its critical point. This fact determines the mobile phase properties (e.g. diffusivity, density, viscosity, etc.) that are intermediate between those of gases and liquids and can be varied and controlled by small changes in the pressure or temperature. The most common fluid used in SFC is carbon dioxide, which has a critical temperature of 31°C, allowing the separation of thermally labile compounds under mild conditions. SFC can be carried out with open tubular and packed columns, with differences in selectivity, detection and need of modifier addition to the carbon dioxide. Both types have been employed in the separation of sterols in a wide variety of samples. Supercritical carbon dioxide has an adequate solvating power for sterol separation with both column types without the need of modifier addition. It is therefore possible to separate sterols in complex samples at lower temperatures than gas chromatography and in shorter times than liquid chromatography.

Importance of the Analysis of Sterols

The analysis of sterols is of great significance from the health point of view and for the quality control of numerous food products.

With respect to quality control of food and nutrition studies, the sterols of vegetable origin (phytosterols) are found in complex mixtures in numerous plants, with the mixture containing some major sterols and a great variety of minor compounds (**Figure 1**). Thus, the sterol profile can be indicative of the origin, or species or variety of food from vascular plants, as well as from fungi or marine organisms. Additionally, these compounds are fundamental in the study of several metabolic pathways.

In the animal world, the variety of sterols is not so broad, and the main constituents are cholesterol and derivative esters. For that reason, supercritical carbon dioxide has been employed for the selective extraction of cholesterol from meat products and edible animal fats, to obtain healthier food for human intake.

On the other hand, some sterol oxides (oxysterols) are known for their toxicity, mutagenicity or carcinogenic properties, a fact that makes the determination of their concentrations in natural matrices very necessary, especially in studies of food quality and physiology.

Characteristics of the Separation of Sterols Using Supercritical Fluids

The main properties of supercritical fluid chromatography which affect sterol separation are related to the high solvating power of supercritical fluids and a low viscosity, which yields a high resolving power and rapid throughput. In addition to its other advantages, the ability of SFC to resolve complex mixtures of low volatility compounds allows the direct injection of samples that contain sterols with no or little pretreatment.

Some sterols can be degraded or lost during exposure to light, heat or extreme values of pH. In the SFC of sterols, all these factors can be avoided, providing a separation under mild conditions that preserves the integrity of the sample.

Finally, the relatively good solubility of compounds with intermediate polarity and volatility such as the sterols has also frequently been utilized in

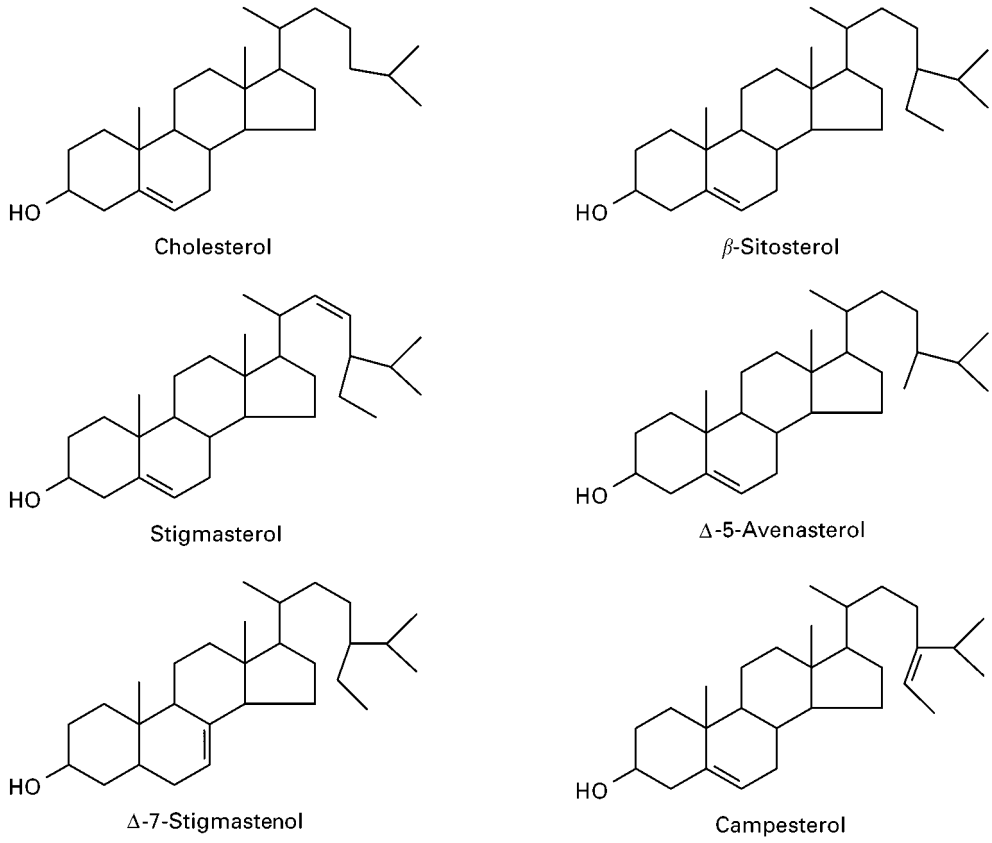

Figure 1 Structures of the main sterols analysed by SFC.

supercritical fluid extraction (SFE) for sample fractionation of the sterols from complex matrices, creating new possibilities for the use of supercritical fluids in multidimensional systems. The solubilities and chromatographic data of the main sterols in supercritical carbon dioxide are well known, and can be found in various books and review articles (see Further Reading).

Isolation of Sterol Subclasses and Sample Preparation

The determination of the sterol fraction in complex matrices such as food provides valuable information on the quality of the product, as well as its purity, origin and plant variety. This analysis presents some difficulties owing to the complexity of the matrix and the relatively low concentration of the sterols in these samples. The most widely used method includes a solvent extraction of the lipid material and isolation of the sterolic fraction, usually after removing the triglycerides through saponification of the lipids and subsequent extraction of the unsaponifiable matter with an organic solvent.

This unsaponifiable fraction contains the sterols and other minor components, such as tocopherols, terpenic alcohols, and hydrocarbons, and therefore often needs to be fractionated. This is conventionally performed by thin-layer chromatography or by solid-phase extraction, prior to high resolution chromatographic analysis.

When the objective is to separate many individual sterols, there is an additional fractionation of the unsaponifiable components after the extraction and sample pretreatment, to isolate the main sterol subclasses (i.e. 4,4-dimethylsterols, 4-methylsterols and 4,4-desmethylsterols, and their esters, oxides and other derivatives), and before the separation of the individual sterols of the subclass of interest. This last fractionation is usually carried out by open column liquid chromatography, thin-layer chromatography, high performance liquid chromatography or more recently supercritical fluid chromatography, after which the sterols can be injected in a gas chromatograph, with or without derivatization.

This whole procedure including saponification, extraction and fractionation is not only time consuming and error prone, and may degrade labile sterols creating artefacts. Consequently, new approaches have been developed in the last few years that avoid several or all these steps by using multidimensional systems or even direct injection into a SFC column.

SFC in Multidimensional Systems

Multidimensional systems take advantages of two different chromatographic separations with complementary characteristics, e.g. one extraction stage and one chromatographic stage. The first step is aimed at producing a clean and undiluted sample containing the fraction of interest, and the second provides a high resolution separation of the target analytes.

It is often the case in chromatography that the largest source of error in the quantitative analysis of sterols and the most time-consuming part of the analytical method is the sample preparation and extraction stage. The main advantages of online systems is that they provide a fast and easily automated sample preparation procedure which reduces or avoids many of the errors of manual extraction. Also, less solvent consumption gives reduced exposure, toxic hazards and lower disposal cost.

The online methods applied to sterol analysis have conventionally consisted in the direct coupling of normal or reversed phase liquid chromatography with capillary gas chromatography (LC–GC), which allows the isolation of the sterolic fraction by LC, followed by online transfer to the gaseous separation in a fast and effective way. An alternative approach is to use packed columns to isolate the sterolic fraction by SFC, as shown recently by Medvedovici *et al*. in 1997, who used a conventional packed column (20 cm × 4.6 mm) with aminopropylsilica gel as stationary phase, for further analysis by capillary gas chromatography–mass spectrometry. This approach can replace the classical preparation methods, yielding a much shorter time with good reproducibility, and it can be automated.

A more interesting approach is the online coupling of SFE and SFC, that allows the transfer of the solutes from the solid matrix to the chromatograph, reducing solvent usage and the need for sample clean-up. This technique has been applied to the separation in open tubular columns of cholesterol in food, and may prove to be of great importance in the future, with the use of packed column SFC; such columns have a large sample capacity and shorter analysis time.

The use of SFC in multidimensional chromatographic systems has a number of advantages. The most common multidimensional system to date, LC–GC, is limited to the determination of thermally stable and volatile solutes, but SFC can replace either the first step (fractionation), or the second step (high resolution chromatography), or even both. In the case of SFE–SFC, the transfer is performed without changes in the mobile phase providing less risk of loss of analytes.

Direct Introduction in SFC

While the analysis of sterol esters does not present difficulties by gas chromatography, a sample preparation step is needed however to remove the fatty material, in order to minimize interferences and protect the GC system. In many cases, the advantage of SFC is that the untreated sample can be injected directly onto a packed column allowing estimation of several lipid fractions at the same time.

Another approach consists in the use of SFC either for fractionation of the oil or for direct selective analysis of the sterol composition of the sample, without previous treatment. This direct injection is a particularly promising technique, and has been applied to the analysis of oils from marine sources to obtain fingerprints of different oil compositions, taking advantage of the very simple sample preparation requirements of SFC.

Separation of Individual Sterols

For the determination of individual sterols, SFC provides the same resolution as gas chromatography and short run times, at temperatures as low as 50–80°C in packed column SFC.

A particular problem is the detection system. Simultaneous detection of many sterols is difficult with ultraviolet detection, as some of the sterols do not possess high absorptivity. Hence this detection mode must often be combined with others to provide a comprehensive detection capability. For this reason, the most usual detection systems for sterol determination are flame ionization detector (FID) or mass spectrometry, which can easily be used with either gas chromatography or SFC.

Separations of sterols have been performed by open tubular column SFC, in samples such as soybean oil derivatives or commercial antioxidant mixtures. Other compounds of interest such as tocopherols, squalene, or even di- and triglycerides can be determined simultaneously (see **Table 1**). (Note that Table 1 is not intended to be a comprehensive review, but aims to provide general information on selected applications.)

Although the most common method for the SFC of free sterols is to employ open tubular columns, separations of sterol-related compounds have also been achieved with columns having different packing materials, ranging from pure silica, to phenyl-, diol-, amino-, or octadecyl-modified silica. For packed columns, the peak symmetry is improved and the retention times of the sterols are shortened when a modifier is added to the mobile phase. This moderates the influence of the free silanol groups of the silica and is analogous to the end-capping of an HPLC silica-based

Table 1 Determination of individual sterols by supercritical fluid chromatography

Column type	*Sample*	*Identified compounds*	*Detector*	*Reference*
Open tubular 10 m × 50 μm i.d. SB methyl 100	Hamster faeces	Free sterols Sterol esters	FID	Pinkston *et al.* (1991) *Journal of High Resolution Chromatography* 14: 401–406
Open tubular 10 m × 100 μm i.d. SB-octyl-50	Soybean oil deodorizer distillate	Free sterols	FID MS/EI	Synder *et al.* (1993) *Journal of the American Oil Chemists Society* 70: 349–354
Open tubular 10 m × 50 μm i.d. SB phenyl 5, 0.25 μm film	Soybean oil condensate	Four sterols, Three tocopherols	FID	Galuba and Gogolewski (1997) *Chemia Analityczna* 42: 245–248

EI, electron impact; MS, mass spectrometer.

stationary phase. In general, the separation of the sterols in SFC is affected by parameters such as the number, location, nature and conformation of the functional groups present in the molecules.

One special case is the determination of cholesterol, which is very often performed on samples with few or no other sterols and where the main objective is to separate this analyte from other non-sterolic lipids and minimize the sample preparation, as will be discussed below.

Determination of Cholesterol and Cholesteryl Esters

In the last few years, the relationship between plasma cholesterol levels and the risk of atherosclerosis and

Table 2 Determination of cholesterol by supercritical fluid chromatography

Column type	*Sample*	*Studied analytes*	*Detector*	*Reference*
Packed column 250 mm × 4.6 mm i.d. particle size 5 μm Kaseisorb ODS-300-5	Human serum	Cholesterol Cholesteryl esters	UV and FID	Nomura *et al.* (1993) *Analytical Chemistry* 65: 1994–1997
Open tubular 10 m × 50 μm i.d. SB-octyl-50, 0.25 μm film	Human serum	Cholesterol Cholesteryl esters	FID	Kim *et al.* (1994) *Journal of Chromatography B* 655: 1–8
Open tubular 20 m × 50 μm i.d. SB phenyl 5	Milk fat	Cholesterol	FID	Huber *et al.* (1995) *Journal of Chromatography A* 715: 333–336
Open tubular 20 m × 100 μm i.d. DB-5	Fish oils	Cholesterol Other lipids	FID	Staby *et al.* (1994) *Journal of the American Oil Chemists Society* 71: 355–359
Open tubular 20 m × 100 μm i.d. DB-5, 0.4 μm film	Marine oils	Cholesterol Cholesteryl esters	FID	Staby *et al.* (1994) *Chromatographia* 39: 697–705
Open tubular 25 m × 100 μm i.d. DB-5, 0.1 μm film	Fish and shark oils	Cholesterol Cholesteryl esters Other lipids	FID	Borch-Jensen and Mollerup (1996) *Chromatographia* 42: 252–258
Open tubular 20 m × 100 μm i.d. DB-5, 0.1 μm film	Shark liver oils	Cholesterol Cholesteryl esters Other lipids	FID	Borch-Jensen *et al.* (1997) *Journal of the American Oil Chemists Society* 74: 497–503

coronary heart disease has been confirmed, resulting in an increase in concern about dietary and blood cholesterol levels. As a consequence, the determination of serum cholesterol is one of the most frequent clinical diagnostic measurements currently undertaken. It is usually performed after hydrolysis, quantifying the total cholesterol by routine enzymatic or colorimetric methods. In many cases, it would be more useful to separately determine free sterols and cholesteryl esters in serum to provide more complete clinical information. This analysis can be carried out by SFC at low temperature, without saponification or derivatization (see **Table 2**). (Again, Table 2 is not a comprehensive review but aims to provide general information on selected applications.) The determination of individual cholesteryl esters cannot be performed by the enzymatic methods available, while gas chromatography requires high temperature to elute the high-molecular-mass unsaturated esters, causing thermal decomposition. Moreover, the detection of cholesterol and related compounds is not very sensitive in HPLC with ultraviolet detection, since free sterols generally have low absorption coefficients. These problems are avoided with SFC in combination with the highly sensitive FID or with mass spectrometry.

Cholesterol Analysis in Human Serum

The analysis of cholesterol in human serum has been performed with both open tubular and packed column SFC, at temperatures of 65 and 45°C, respectively. With open tubular columns, it is possible to determine quantitatively free, total and individual esterified cholesterol with a simple liquid–liquid extraction without derivatization. The use of FID gives detection limits of 4–6 pg. The quantitative results for the analysis of total cholesterol in reference sera and real samples show good agreement between the SFC, GC (with derivatization), and enzymatic methods.

With columns packed with inert octadecylsilica, there is no need to add any modifier to the carbon dioxide, allowing the simultaneous use of ultraviolet and FID. In addition, the use of carbon dioxide as the supercritical fluid allows ultraviolet detection at wavelengths as low as 190 nm, which are usually below the practical limit with HPLC because of the general absorption of most solvents at this

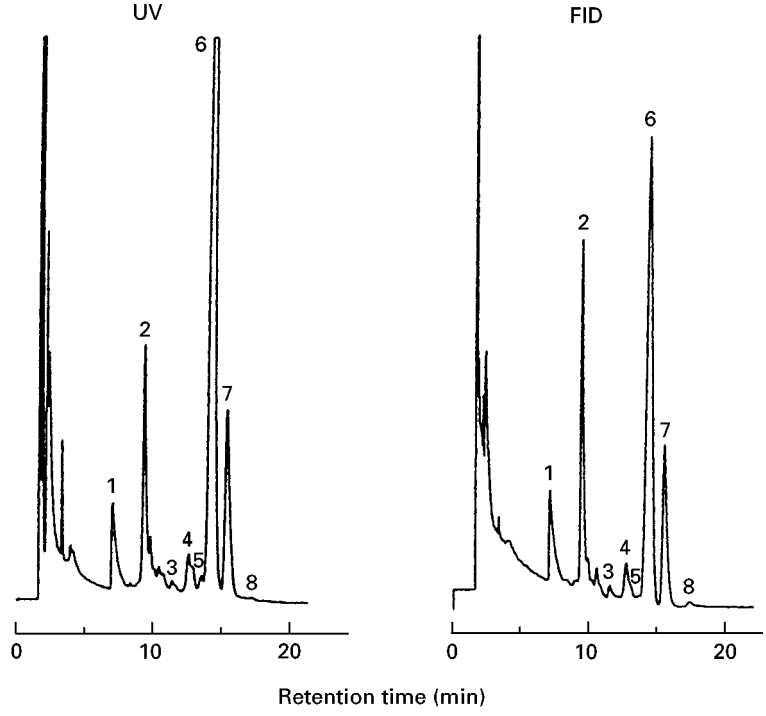

Figure 2 Chromatograms of cholesterol and cholesteryl esters from human serum reference material with ultraviolet (UV) (wavelength 190 nm) and flame ionization detection (FID). Peak identification: (1) cholesterol, (2) cholesteryl laurate (internal standard), (3) cholesteryl myristate, (4) cholesteryl palmitoleate, (5) cholesteryl linolenate, (6) cholesteryl palmitate + cholesteryl linoleate + cholesteryl arachidonate, (7) cholesteryl oleate, (8) cholesteryl stearate. Chromatographic conditions: reversed-phase HPLC column (250 mm × 4.6 mm i.d.; particle size, 5 μm), column temperature 45°C, CO_2 pressure 200 atm, CO_2 flow rate 750 mL min^{-1} at room temperature under 760 mmHg. (Reproduced from Nomura *et al.* (1993) *Analytical Chemistry* 65: 1994–1997, with kind permission from the authors and the publisher. Copyright 1993 American Chemical Society.)

wavelength. Use of lower wavelengths in ultraviolet detection results in a greater sensitivity than higher wavelengths for all cholesterol and cholesteryl esters. Both ultraviolet and FID showed good agreement in a study of cholesterol and their esters, with a detection limit of 20 ng for cholesterol and cholesteryl palmitate, though better reproducibility was obtained with UV detection (**Figure 2**).

Supercritical fluid chromatography can be a useful tool in studies on cholesterol and cholesteryl ester metabolism in serum and biological fluids in general, though some improvements in the selectivity are still needed to properly resolve some difficult pairs of steryl esters.

Cholesterol Analysis in Edible Oils

There are numerous applications of SFC with capillary columns for the determination of components of oils of marine animals especially from Mollerup's group at the Technical University of Denmark (see Table 2). In these samples, numerous lipids have been analysed including cholesterol and its esters, together with vitamin E, squalene, and di- and triglycerides. Open tubular column SFC is therefore very convenient for these complex samples owing to its high resolution and relatively low analysis temperature (150–170°C versus 250–300°C for GC). For example, the analysis of cholesterol and cholesteryl esters in seafood, shark liver, seal and other fish oils has been accomplished with open tubular column SFC both with direct injection of the diluted oil and with previous saponification and fractionation by thin-layer chromatography (see Table 2). The latter purification avoids the overloading of the open tubular columns (100 μm internal diameter) by the squalene that is present in high concentrations in these samples. Saponification followed by fractionation is, however, time consuming and the recovery of minor components can be difficult. With direct injection in SFC, simultaneous determination of cholesterols, triglycerides and other lipids has been achieved, with enhanced separation power compared with HPLC, while gas chromatography always requires sample pretreatment of these oils to ensure chemical stability at high temperatures.

In other solid and more heterogeneous foods, sample preparation is the most time-consuming step in the routine determination of, for instance, cholesterol levels in daily diet and remains the largest source of error in the quantitative analysis of sterols.

Future Trends

Current developments in new types of columns, equipment and detectors for SFC show that this technique is still developing and expanding and will achieve greater use in the future, particularly with the advent of new chromatographs for packed capillary columns and with automation of modifier addition, which will be very valuable in the determination of more polar sterols and their oxides in samples where high resolution and mild conditions are imperative.

Another important development is the more frequent use of solvents under subcritical conditions which, in practice, is eliminating the rather artificial boundary between SFC and liquid chromatography.

One of the main advantages of using packed capillary columns over conventional packed columns is the improved performance when this separation is coupled to mass spectrometry, thus providing structural determination of the sterols. This is expected to be especially important in the coming years owing to the development of new commercial equipment for SFC–MS.

Another potential source of improvement is the use of new detectors with higher sensitivity than ultraviolet but compatible with the use of modifiers in packed capillary column SFC. The amperometric detector is a good example.

These anticipated developments in SFC technology will be important in the field of sterol analysis, though an automated SFE–SFC without tedious sample preparation and large solvent consumption would be one of the most valuable future developments for sterol analysis.

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Thin-Layer (Planar) Chromatography

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Sterols are steroid compounds widely distributed in various biological materials, e.g. variety plant and animal lipids, medications, food and dietary supplements. They are basic metabolites in living organisms and they are also precursors of a variety of bile acids, provitamins and steroid hormones. Therefore the analysis of sterols is important in biochemistry, medicine and pharmacy. There is considerable interest in the study of the relationship of plasma cholesterol concentrations to the risk of developing coronary artery disease. Determination of phytosterols and cholesterol is important for the diagnosis of phytosterolaemia and in dietary treatment of hypercholesterolaemia.

The collective name for sterols has been adopted for all naturally occurring crystalline unsaponifiable steroid alcohols. The basic sterol is 5α-cholestane, and the structure numbering system for sterols are given in **Figure 1**. In general, these compounds are 3-monohydroxysteroids, having 27, 28 or 29 carbon atoms and nearly all have one or more double bonds. The double bond is most commonly found at position 5, with double bonds at C_7 and C_{22} also being prevalent. Sterols are classified into five groups: cholesterol and its companions, zoosterols, phytosterols, mycosterols and vitamin D. Examples of sterols from each group are given in **Table 1**.

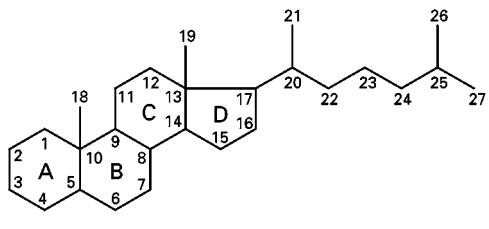

Figure 1 5α-Cholestane skeleton and numbering system for sterols.

There are four principal methodologies used in sterol chromatography: gravity flow column liquid chromatography (GCC), high performance liquid chromatography (HPLC), gas chromatography (GC) and thin-layer chromatography (TLC). For its selectivity, sensitivity and efficiency, TLC is one of the most frequently employed procedures for the separation of sterols, both for their characterization and for their quantitative analysis.

Preparation of Sample

Since sterols are present in different materials, sample preparation is a very important part of their analysis.

The first step is an extraction procedure which is performed either directly on the previously deproteinized sample or after cleavage of any conjugates present. Diethyl ether, dichloromethane, ethyl acetate, chloroform and other medium polarity organic solvents can be used for extraction.

The next step is purification of the extract or, more exactly, separation of sterols from other lipids, usually by TLC on a silica gel G plate using an *n*-heptane–diethyl ether–acetic acid (85 : 15 : 1) mixture as the mobile phase. Under these conditions the cholesterol and phytosterols are concentrated in one band. Separation and quantitative analysis of individual sterols are performed after elution from the plate by GC, TLC or some other technique.

Some sterols (e.g. vitamin D) are sensitive to atmospheric oxygen, traces of acids and bases, light and heat. Therefore, all steps should be carried out in a cool place, protected from exposure to direct light, and only highly purified solvents should be used.

Stationary Phases and Solvent Systems

Generally, the chromatographic separation of individual sterols is difficult due to the large number of epimers and unsaturated isomers. Various forms of silica gel are most frequently used in the TLC of