Improved SFE Recovery of Trace Analytes from Liver Using an Integral Micrometering Valve – SPE Column Holder Assembly

A novel integral restrictor–collector has been designed for use with a conventional supercritical fluid extraction (SFE) apparatus. The assembly reduces the path length between a micrometering valve and collector (a solid phase extraction (SPE) column), obviating the need for the complicated tubing and connectors usually associated with such devices. Also described is a heating-block assembly which encases the micrometering valve and provides uniform heating of the valve during extraction.

The valve–SPE column assembly was part of a system used to perform the first reported SFE multi-residue drug recovery from fortified liver. Extractions used carbon dioxide pressurized to 690 bar as the supercritical fluid. Flow rates of expanded gas through the SPE columns were 3–4 L/min with concomitant quantitative trapping of the analytes on the sorbent bed. After SFE the three nitrobenzamide antimicrobial drug residues from the liver were eluted from the SPE columns by off-line analysis. The results demonstrated that losses of trace level analytes in tissue may be significantly reduced by including an integral metering valve–collector assembly as part of the SFE apparatus.

1 Introduction

The levels of veterinary antibiotic and antimicrobial drug residues in animal tissue after slaughter are monitored by the Food Safety Inspection Service (FSIS) of the USDA [1]. Their regulatory laboratories employ various methods of extraction to isolate these residues from sample tissues prior to the assay. In order to reduce the use of organic solvents and improve sample through-put, alternatives to conventional solvent-type sample preparation methods are being sought. For this reason, supercritical fluid extraction (SFE) is under investigation in this laboratory to determine its potential applicability to replace selected solvent methods for the isolation of polar analytes from animal tissue.

Drug residues in tissue are generally detected in the low ppm to ppb range. Given the low levels of these compounds in the target tissue, a SFE procedure must be capable of recovering analytes with minimum losses as a result of instrument design. One problem which must be considered when designing an SFE method for isolation of drug residues from tissue is that most tissue samples contain various levels of fat, which, because of its relatively high solubility in supercritical carbon dioxide [2] is co-extracted with the analyte of interest. All the extracted fat containing target analytes must be recovered quantitatively to ensure complete drug residue recovery.

In most SFE apparatus, the potential for loss of an extracted drug residue/fat mixture is greatest at the restrictor – collector interface. In order to study the minimization of solute losses at this point, we have designed two unique integral micrometering valve – collector assemblies. In our earlier versions of the instrument design, the collector was a laboratory-assembled, solid-phase column device which was packed with sorbent by hand. The collection device was positioned several centimeters downstream of the restrictor. Poor analyte recovery was observed with this system, apparently because of analyte precipitation between the restrictor and collector. To overcome this problem, and to enable the use of prepacked SPE columns, the integral micrometering valve–SPE column holder assemblies were designed for use with commercially available, off-the-shelf SPE columns which are quickly removed from the assemblies for off-line analysis. Compared with results obtained from a conventionally attached collection device, dramatic improvements in the recovery of three nitrobenzamide antimicrobial drugs from fortified liver were observed when either of these assemblies was used in the SFE apparatus.

2 Experimental

Extractions were performed on a laboratory-assembled SFE apparatus shown schematically in **Figure 1**. Carbon dioxide was pressurized by adjusting the air intake valve of a gas booster

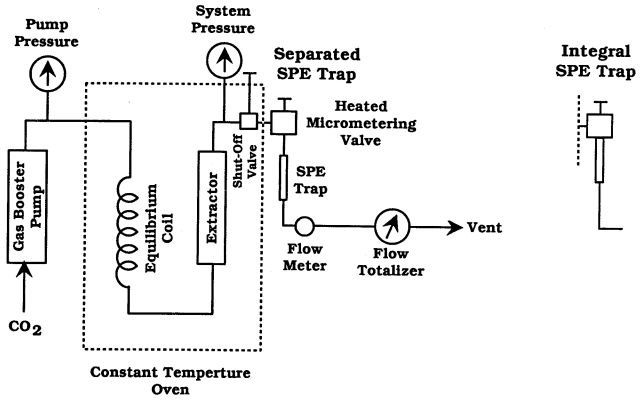

Figure 1

Schematic diagram of SFE apparatus equipped with separate and integral micrometering valve – SPE column holder assemblies.

pump (Haskel Engineering, Burbank, CA, USA; Model AGT-62/152C). Pump pressure was monitored by means of a pressure gauge. The pressurized fluid flowed into a 3 m heat transfer coil mounted in a Varian (Sunnyvale, CA, USA) model 2100 gas chromatograph oven. The fluid, at equilibrium temperature, next flowed into an extractor tube containing fortified liver (1 g) blended with Hydromatrix (4 g; Varian-Analytichem, Harbor City, CA, USA), mounted vertically in the oven. This extractor (volume 26 mL) was assembled from a nipple (Autoclave Engineering, Erie, PA, USA; part no. CNLX-1208) and two connector nuts (Autoclave; part no. 20F12463). A pressure transducer (Carlin Associates, Rock Island, IL, USA; GP:50 model 311-C) was placed in-line with the extractor to monitor pressure drops across the extraction vessel. Beyond the pressure transducer was a shut-off valve (Autoclave; model 10V2021) mounted in the oven with an extended handle accessible outside.

This valve was used to stop the flow of fluid during the equilibration period. When the valve was opened, the fluid was directed to a micrometering valve (Autoclave; model 10VRMM2812) encased in an aluminum housing (see below) and bracketed to the exterior of the oven wall. This valve controlled the flow rate of the expanded gas through the SPE column, which was connected to the valve through two pathways as shown in Figure 1. Gas flow was measured downstream using a digital flowmeter (McMillian, Georgetown, TX, USA; model 110) and the total gas flow was recorded on a gas totalizer (American Meter Co., Philadelphia, PA, USA; model DTM-115). Three versions of the heated micrometering valve – SPE column assembly were fabricated as described in the following sections.

2.1 Heating Block for Micrometering Valve

A schematic drawing of the aluminum block heating assembly is depicted in **Figure 2**. This assembly was constructed using two sections of aluminum bar stock each 3.5 cm (width) × 3.9 cm (height) × 1.9 cm (depth). Each half was hollowed to fit the exterior of the micrometering valve and the two sections were sandwiched over the valve to form a solid block. The assembly was bolted together through the holes provided in the micrometering valve and anchored to an L-shaped bracket on the side of the oven.

Two additional holes were drilled into the heating block assembly: a 1/4″ hole, positioned near the center, was drilled in one of the two halves to accommodate the cartridge heater (Watlow Electric, St Louis, MO, USA; E2A57) and a second hole was drilled through the top of the aluminum block and into the micrometering valve to contain the thermocouple (Omega Engineering, Stamford, CT, USA; TJ60-CASS-116-G). This hole (1/16″) was drilled to

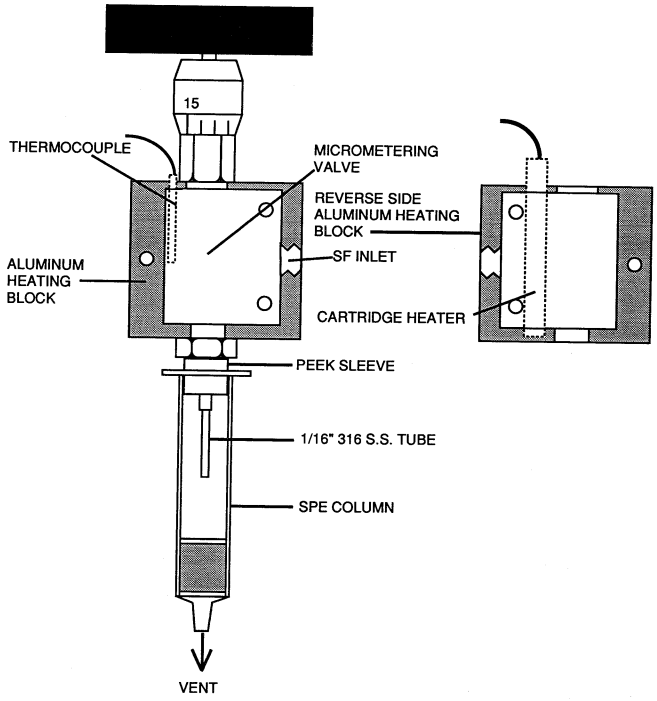

Figure 2

Schematic diagram of integral heating block–micrometering valve–SPE column holder assembly showing attachment of SPE column to seat retainer nut over PEEK sleeve.

a depth of 2.4 cm and positioned so that the hole in the micrometering valve was 2.5 mm from the front and side edges of the valve block. After positioning the heating cartridge and the thermocouple in the aluminum housing and valve, respectively, they were then connected to the temperature controller (Omega; CN4401TR-D).

Any of the three seat retainer designs described below may be used to interface SPE columns to the heating block–micrometering valve assembly.

2.2 Original Seat Retainer Nut – Column Assembly

This method of attaching an SPE column to a valve used the seat retainer nut which is part of the original Autoclave micrometering valve assembly. To make the connection to the seat retainer nut, the female connector of an HPLC guard column holder (Upchurch Scientific, Oak Harbor, WA, USA; part no. C-100) was drilled to accommodate a length of 1/8″ stainless steel tubing (**Figure 3a**). One end of the tubing was cut to a flat tip, inserted into the column holder to a depth of 3.6 cm, and brazed. The other end of the 1/8″ tube was fitted with an Autoclave nut and ferrule and attached to the valve through the seat retainer nut.

A silicone washer, 1/16″ thick, was cut to the proper dimensions and inserted into the column nut. To attach the SPE column to the column holder, a retaining nut was fabricated from aluminum rod. The retaining nut could be threaded into the column nut, making contact with the flange of the polypropylene SPE column (Applied Separations, Lehigh Valley, PA, USA), which was in turn compressed against the silicone washer. This arrangement ensured a gas-tight seal.

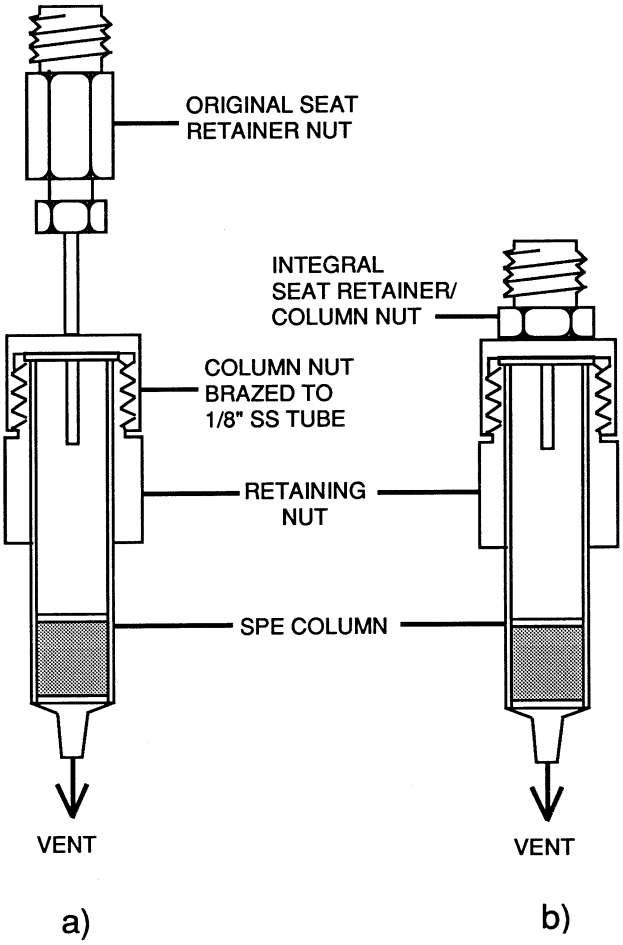

Figure 3

Schematic diagrams of (a) separated valve seat retainer nut attached to guard column holder nut *via* 1/8″ tubing; and (b) guard column holder nut directly attached to re-fabricated seat retainer nut.

The flange on commercial 6 mL SPE columns is oval rather than circular. Prior to insertion into this assembly, the flanges on the 6 mL columns were, therefore, trimmed to a circular shape, leaving enough flange to engage the retaining nut. The standard 3 mL SPE columns are the same diameter as the column holder and could be inserted into the nut without modification.

2.3 Integral Seat Retainer – Column Nut

This assembly is shown in **Figure 3b**: it differs from 3a only in that the seat retainer was attached directly to the column nut. The original seat retainer nut could be machined to make this piece, or a new nut could be fabricated from 316 grade stainless steel stock using the original specifications for the valve end of the nut. When the original nut was used for this purpose, a 5 mm minimum section of hexagonal nut was retained, and the lower section was cut leaving enough of this section to form a plug and socket connection in the column holder nut. The lower section was then rounded, and a section of 1/8″ stainless steel tubing with a flat tip was brazed to the nut. The modified nut was inserted into the column holder nut through an opening 0.001–0.003″ wider on each side than the diameter of the modified seat retainer nut and brazed to form an integral unit.

2.4 Integral Seat Retainer – PEEK Sleeve Nut

A schematic drawing of this nut attached to the heating block – micrometering valve assembly is shown in Figure 2. This unit was fabricated from a 316 stainless steel rod 3.5 cm long. The threaded section was machined to conform with the specifications of the original seat retainer nut. Below the hexagonal nut section, which was 6 mm wide, the stock was machined to a diameter of 11.5 mm and a length of 8 mm. Below this section, the stock was further reduced to 4 mm in diameter by 5 mm long. A sleeve, 13 mm diameter by 8 mm, was fabricated from a PEEK rod (polyether-etherketone; Greene, Tweed, and Co., Hatfield, PA, USA). The PEEK sleeve was fixed to the 11.5 mm diameter section of the nut with two-part epoxy resin. A 1/16″ hole was drilled through the nut assembly, and a length of 316 stainless steel tubing, 5.5 cm long × 0.03″ i.d., with a flat tip at one end, was inserted the length of the assembly and brazed in place.

3 Results and Discussion

The heating block – micrometering valve assembly was designed to overcome a problem inherent in the use of restrictors to decompress supercritical fluids, *i.e.*, flow rate changes and possible deposition of analyte within the restrictor as a result of uneven or non-uniform heating of the latter. These phenomena were observed when attempts were made to control the temperature of the micrometering valve by wrapping it with heating tape and/or heating its surface with a heating gun. Use of these devices was inadequate to control flow rate or prevent deposition of analyte and co-extracted fat within the valve and tubing at the flow rates (3–4 L/min) of expanded gas (carbon dioxide) required to extract nitrobenzamide antimicrobial drugs from liver tissue. We have found that flow rates of this magnitude are needed for effective isolation of many classes of antibiotic and antimicrobial drug from animal tissue. Such flow rates are much higher than those used by other investigators to isolate drugs from other matrices [3, 4], which in turn places limits on the type of restrictor which may be used in SFE systems employed for this application.

The heating block assembly for the micrometering valve was easily constructed from aluminum bar stock. The thermocouple for this unit was placed in a hole drilled in the micrometering valve housing near the valve stem where the supercritical fluid expands to a gas. Even at the high flow rates used in these experiments, the valve temperature could be maintained to within ±1° of the controller set point.

Many SFE systems are designed to vent solute into a flask or a vial filled with solvent. Unfortunately, these approaches may require dilution of the analytes and may lead to the loss of analyte owing to incomplete trapping. Drug residues in animal tissue are generally present in the ppb-ppm range, a level which requires that precautions be taken to ensure that losses are minimized

during the trapping process. For this reason, it was decided to develop a method of focusing the recovered drug residues contained in the co-extracted fat on sorbent beds from which the analytes of interest could be eluted with a minimum amount of solvent, rather than employing other trapping methods.

Our initial studies were performed using an assembly of the type shown in Figure 3a. The SPE column containing the sorbent, inserted in the column holder, was connected through a section of tubing to the seat retainer nut of the micrometering valve. The analyte/fat mixture extracted from liver tissue was, as it left the tubing tip, trapped on the SPE column walls, and on the frit compressed on top of the sorbent bed. No back-pressure problems were encountered when the carbon dioxide stream passed through the sorbent bed, despite gas flows of 3–4 L/min. Such high flow rates could, in theory, result in analyte loss as compounds are swept through the sorbent bed before adsorption occurs. To test this proposition, an adapter was used to attach a second SPE column to the Luer connection on the first. Irrespective of the total amount of carbon dioxide gas which passed over the sorbent bed (up to 150 L) none of the target analytes (4 μg each in the fortified liver samples) was detected in the second SPE column.

A review of various off-line trapping methods for SFE by *Mulcahey et al.* [5] suggests that a solid phase sorbent may provide two trapping mechanisms – cryogenic and adsorption. The examples given in that report were compounds such as PCBs and phenols. Whereas cryogenic trapping may be required for such compounds, it is not necessary for analytes such as nitrobenzamide antimicrobial drugs contained in fat matrices. In this instance, cooling of the SPE column resulted in reduced recovery of the target analytes, perhaps because the lower temperatures cause water extracted from the liver to be condensed on the sorbent bed (neutral alumina) thus changing the activity of the solid phase packing. In this study, SPE columns were used at ambient temperatures. At the operating temperature of the micrometering valve (110 °C), however, the temperature of the carbon dioxide gas leaving the tubing tip was between 50 and 60 °C at the set flow rate of 3 L/min; this slightly elevated the temperature of the sorbent bed during the course of the extraction (20 min).

Although this valve assembly (Figure 3a) was efficient at trapping analytes on the sorbent bed, analyte losses occurred owing to the method used to attach the SPE column holder to the seat retainer nut. The section of 1/8″ tubing, plus the additional connections, resulted in analytes being retained on the tubing walls instead of being swept from the plumbing on to the sorbent bed.

This phenomenon is illustrated by the results shown in **Table 1**. A mixture of three nitrobenzamide antimicrobial drugs, aklomide, nitromide, and zoalene (4 μg of each) was added to liver. They were extracted with supercritical carbon dioxide at an oven temperature of 60 °C and an expanded gas flow rate of 3 L/min. In separate experiments, the recovered solute mixtures were collected on SPE traps by passage of 50 L, 100 L, and 150 L of carbon dioxide, respectively. After each experiment, the extracted sample matrix was re-extracted using a solvent method [6] to determine the amount of each drug remaining in the sample matrix. We found that the drugs had been exhaustively extracted after passage of 50 L of carbon dioxide, indicating that the increased recoveries resulting from use of increasing amounts of carbon dioxide were a result of system plumbing, rather than incomplete extraction; more specifically they were a consequence of the design of the valve – collector interface.

To minimize solute loss in the valve – collector interface, the integral seat retainer – column nut assembly shown in Figure 3b was fabricated (Section 2.3). This design differed from that shown in Figure 3a in that the tubing and connectors between the seat retainer and the column nut were eliminated. The path length from the point of decompression in the valve to the tip of the tubing was reduced from 9 to 4.5 cm. That this reduction in path length influenced recoveries of drug residues is apparent from the data in Table 1. The maximum recovery of the three antimicrobial drugs using the separated valve – column assembly (Figure 3a) occurred after passage of 150 L of carbon dioxide passed over the sorbent bed. Using the integral valve – column assembly (Figure 3b), significantly higher recoveries of these compounds were obtained after a total flow of only 60 L of carbon dioxide. These differences are a result of the reduction in the length of the interface since all other system variables were unchanged.

The 1/8″ tubing used in the above two designs was 316 stainless steel. We also designed and fabricated a third type of seat retainer – SPE column interface incorporating 1/16″ stainless steel tubing into the assembly (Figure 2). The specifications of this unit, machined from 316 stainless steel stock, were the same from the valve end to the hexagonal nut as the assembly shown in Figure 3b. The column end of the assembly used an oversized PEEK sleeve, attached to the steel body with epoxy resin, to hold the 6 mL SPE column on the assembly in a leak-tight seal. The PEEK sleeve was a necessary addition to this design, because polypropylene SPE columns soften and loose their seals when they are in contact with a heated metal surface at the valve operating temperatures used (>110 °C).

The PEEK sleeve on the assembly insulated the column from the heated metal surfaces and ensured a tight seal throughout the experiment. Since no column holder or retaining nut was needed with this design, it was not necessary to remove part of the SPE column flange in order to slide the column into a holder. Since, moreover, with this design the SPE column did not require a flange to provide a seal, the columns could be cut to any length and fitted on the PEEK sleeve. Using this concept, the 316 stainless steel tube could be placed at any distance above the sorbent bed, dependent only on the requirements of the specific application.

Table 1

Recovery of three nitrobenzamide antimicrobial drugs from SPE columns[a] attached to separated and integral valve – collector assemblies.

	Liters of expanded carbon dioxide			
	Separated[b] SPE Column			Integral[c] SPE Column
	50 L	100 L	150 L	60 L
Aklomide	50	71	86	96
Nitromide	41	55	71	82
Zoalene	52	70	83	88

[a] sorbent bed: 1.5 g neutral alumina
[b] *cf.* Figure 3a
[c] *cf.* Figures 2 and 3b

The integral seat retainer–PEEK sleeve nut (Figure 2) was tested, again using the antimicrobial drugs listed in Table 1. Results obtained were similar to those found when the integral seat retainer – column nut assembly (Figure 3b) was used in the micrometering valve. Since either modification gives the same results, they may be used interchangeably depending on the user's application.

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Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture in preference to others of a similar nature not mentioned.

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Splitless Injection of Large Volumes: Improved Carrier Gas Regulation System

Summary

The classical vaporizing injector has been modified for splitless injection of large volumes: during solvent evaporation in the packed vaporizing chamber, the carrier gas supply is interrupted and the septum purge outlet fully opened. This prevents vapors penetrating the gas regulation system and keeps the pressure increase in the injector to a minimum.

1 Introduction

Sample volumes injected by the splitless technique can be increased beyond 100 µl provided no volatile solutes are to be analyzed [1]. The concept is based on the principle of vapor overflow and on cooling of the vaporization zone in the hot injector to the solvent boiling point by the evaporating sample. Expansion of solvent vapors backwards from the injector insert and their removal through the septum purge exit is acceptable because all except the volatile components are retained at the cooled site of evaporation.

When solvent evaporation is complete, *i.e.* cooling by solvent evaporation ceases, the temperature returns to that of the injector; the solutes are then vaporized and transferred into the column. The vaporizing chamber must be packed in order to provide a stationary phase for both retaining the solute material at the cooled site of evaporation and keeping the sample liquid in position (resisting the strong current of escaping vapors). Evaporation of 200 µl of solvent has been found to take some 2–5 s [2].

The technique cannot compete with large volume on-column injection in terms of accuracy, but its application is simple and nearly all of the solvent is vented, protecting the detector. It is, furthermore, expected to be highly resistant to contamination.

The proposed technique presupposes separated split and septum purge exits in order to enable closure of the split exit (splitless injection), while the septum purge is fully open. Using the injector of the Carlo Erba/Fisons series 4000–6000 instruments without modification and packing the 4 mm i.d. insert with Tenax, at least 200 µl of solutions in organic solvents could be injected. 500 µl injections had to be performed at reduced speed, and 1 ml injections required sample introduction over a period of at least 30 s, otherwise vapors penetrated too far into the carrier gas supply system, leading to an extremely broad solvent peak.

The conventional injection system is suitable for a wide range of applications. For injection of extremely large volumes, however, including samples, such as aqueous samples, creating large volumes of vapor, the carrier gas supply system can be improved, as shown in this paper.

2 Principles of the Improvement

There are two aspects of the improvement: arresting the supply of carrier gas and complete opening of the septum purge outlet during solvent evaporation. Although each is an independent goal, they are technically related.

Stopped flow injection has been proposed by *Bayer* and *Liu* [3], although for another purpose: to allow more time for sample evaporation in split injection. For splitless injection of large volumes, interruption of the carrier gas supply during solvent evaporation is advantageous for two reasons. Firstly, it eliminates the danger of solvent vapors penetrating the carrier gas line as far as the manometer and the pressure regulator, contaminating the latter (the reason injection of volumes >400 µl had to be performed slowly). Secondly, retention of the volatile solutes is improved by reducing the temperature at the site of evaporation: as this temperature corresponds to the solvent boiling point at the current pressure, we are interested in keeping the pressure low, *i.e.* in eliminating the carrier gas inlet pressure.

The septum purge outlet (vapor exit) is opened as widely as possible in order to release the solvent vapors with a minimum of back-pressure. If, *e.g.*, 500 µl of water is evaporated within 10 s, *ca* 700 ml of vapor is formed at a rate of more than 4000 ml/min. Only wide-bore escape routes release it without significant back-pressure.

Opening of the vapor outlet presupposes interruption of the carrier gas supply. The septum purge outlet must be switched back to low flow when the supply of carrier gas is restored. To this end, the two modifications are interrelated: the carrier gas must be turned off when the vapor outlet is opened, and this outlet must be closed when the carrier gas is turned on again to transfer solute material into the column.

3 Switching by Rotating Valve

Experiments were performed using a new Carlo Erba/Fisons injector, as installed on the 8000 and Mega 2 series gas chromatographs. The 5 mm i.d. liner for splitless injection, with a taper at the bottom to provide a vaporizing chamber 80 mm long, was