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Review

Solid-phase extraction technique for the analysis of biological samples

T.R. KRISHNAN*† and I. IBRAHAM‡

†*School of Pharmacy, Memorial University of Newfoundland, St John's, Newfoundland, Canada A1B 3V6*
‡*College of Pharmacy, Dalhousie University, Halifax, Nova Scotia, Canada B3H 3J5*

Abstract: This article reviews the literature on solid-phase extraction published in the last 10 years. Emphasis has been placed on dealing with samples of biological origin. The sections consist of introduction, history and development, types of columns, selection of a suitable column, types of samples, advantages and applications.

Keywords: *Solid-phase extraction; liquid–solid extraction; sample preparation; biological sample.*

Introduction

In the last two decades instrumentation techniques to analyse xenobiotics and other chemical substances, have made remarkable progress. With the advent of microprocessors and computers, analytical techniques have become easier to perform and automation has become feasible. Robotics has found a place in analytical laboratories where hundreds of samples can be analysed without much human supervision. Despite all the sophistication, until the recent past, test samples, especially those of biological origin, needed an elaborate and labour intensive sample preparation procedure, to remove unwanted substances such as cells, proteins, albumin, water, etc. from blood, urine and other biological samples. As a result, the sample preparation step adversely affected the efficiency of the entire analytical procedure.

Among the various analytical instruments, liquid chromatography (LC) has emerged as one of the most accepted and widely employed techniques, due to its high efficiency, reliability and versatility in being able to be combined with other tools, such as infrared and mass spectrometers. Advancement in HPLC technology made even greater strides with the introduction of bonded silica as the stationary phase. The remarkable success in HPLC technology encouraged researchers to apply the principles of chromatography, to separate the analytes of interest from other interfering substances, while treating test samples. Small columns containing 100–500 mg of packing material were used to separate and selectively elute analytes from unwanted substances. This technique is known as solid-phase extraction (SPE) or liquid–solid extraction.

Solid-phase extraction, which is now emerging as a very important sample preparation technique, is preferred to other traditional extraction procedures, such as liquid–liquid extraction (LLE), mainly because it is more efficient and much less time-consuming. By integrating SPE with HPLC complete automation of sample analysis can now be achieved. The increase in the number of published articles from about two in 1982, to about 72 in 1989, is proof of SPE's increased acceptance. Since reports describing SPE are either analytical surveys [1] or only a part of an article on general methods for sample preparation [2], a detailed review specifically on the SPE technology is deemed appropriate.

History and Development of SPE

The history of using solid phases to isolate drugs from biological samples dates back to 1923 when Whitehorn [3] used permutite and

*Author to whom correspondence should be addressed.

then subsequently silicic acid [4] for extracting adrenaline. In the 1950s, Lund *et al.* [5] used alumina columns for extracting adrenaline and noradrenaline from blood samples. Then Bergström and Hansson [6] successfully extracted catechol bases, adrenaline and histamine from crude extracts of glands, using Amberlite IRC-50. Later Bertler *et al.* [7] developed a method to extract catechol amines from tissues using cation exchange resin, Dowes 50. By the mid 1960s more complex samples were being tried. Dole *et al.* [8] developed a method, using cation exchange paper chromatography, to detect a number of narcotics, tranquilizers, amphetamines and barbiturates from urine samples but the method was more qualitative in nature. In the 1970s, the stress was to develop techniques which were more sensitive. Fujimoto and Wang [9] used Amberlite XAD-2 resin columns to quantify narcotic analgesics from urine and could determine as low as 0.6 mg ml^{-1} of urine. It is noted that, in most of the above cases, the principle of ion exchange was used to separate drugs from biological samples. Subsequently, Meola and Vanko [10] tried the adsorption phenomenon using charcoal to concentrate a number of drugs (barbiturate, glutathimide, ethchlorvynol, amphetamine, phenothiazine, quinine, morphine, cocaine and its metabolites) from urine and achieved an average detection limit of 1 mg ml^{-1} of urine.

By the late 1970s HPLC technology had made rapid progress and one of the major developments was the use of silica and bonded silica as the stationary phase. Waters Associates and Analytichem International were among the first to develop the concept of using sorbents, similar to those used in HPLC, packed in miniature columns, to isolate drugs and chemicals from other interfering impurities of test samples. Specifically, small disposable cartridges containing silica, bonded silica and other phases were developed for commercial use and these were called SPE columns. Narasimhachari [11] evaluated C_{18} SepPak® columns (Waters Associates) to extract tricyclic antidepressants from biological samples. The advantages of a SPE procedure included, higher recovery of analytes leading to an improved precision of analysis, economy of solvent, labour and time. More manufacturers of laboratory-supplies have entered the SPE column market and, currently, there are more than 10 major sources from which to choose. SPE columns with attached sleeves are also available, which can be used in robotic analytical technique. Among other companies DuPont, Varian Sample Preparation Products (formerly Analytichem International), Waters Associates, and Gilson and Zymark have developed automated sample processors which use the principle of SPE. Predictably the SPE technique is soon to become an integrated part of the analysis, separating chemical compounds of interest from interfering and unwanted substances, of test samples.

Types of Solid-phase Extraction Columns

The disposable SPE column body is generally made of polypropylene. It is either cylindrical or conical in shape, with a male luer hub on one end, which can be fitted to an extraction device. A portion of the column body is filled with the solid sorbent, of particle size usually about 40 μm, sandwiched between two polyethylene frits [12]. The sample is applied to the top of the column and drawn through the sorbent by applying a negative or positive pressure or by using a centrifugal force. Negative pressure can be applied by using either a syringe, via a short piece of plastic tubing (⅛" i.d.), or a commercially available vacuum manifold. Such a manifold can fit up to 24 columns and is very advantageous when a large number of samples are to be handled. A conical shaped disposable SPE column can accommodate up to 15 ml of sample. An accessory reservoir can be attached, if necessary, to the top of the column to further increase its void volume.

A wide range of columns with different packing sorbent beds are available. They can be categorized in the following way. Adsorption phase: Silicagel (SiOH), florisil (MgSiO), alumina (Al_2O_3) and Kieselguhr (SiOn). Normal phase: amino (NH_2, cyano (CN) and diol (COHCOH). Reversed phase: methyl (C_1), ethyl (C_2), hexyl (C_6), octyl (C_8), octadecyl (C_{18}), cyano (CN), cyclohexyl (C_6H_{11}) and phenyl (C_6H_5). Anion exchange: quaternary amine (N^+), amino (NH_2) and diamino (NH_2–NH). Cation exchange: aromatic sulphonic ($C_6H_5SO_3H$) and carboxylic acid (COOH).

The above list is not meant to be exhaustive in covering all the commercially available SPE columns. The reader can find a more detailed list in the laboratory supplies-manufacturer's

catalogues. Many manufacturers have SPE columns under specialized categories, such as biochromatographic, environmental, narcotic drug-abuse testing, etc. Most manufacturers provide a 'method development kit' which has a few columns from different categories. Such a kit is ideal for developing a suitable extraction procedure for a new compound.

Selection of a Suitable Column

Basically, there are two different approaches for choosing a suitable sorbent. One can either choose a sorbent that has a relatively higher affinity for the analyte, to retain it, and let go the unwanted components of the sample or choose a sorbent that has a low affinity for the analyte, allowing it to pass, while retaining the unwanted components. In the former approach, the analyte has to be later eluted out with the help of a suitable eluent, while in the latter, no elution step is needed. In SPE, the principles of selective adsorption, partition or both are involved and hence both the nature of the sorbent and the sample play important rôles. The pH of the sample and the sorbent bed are also important since, they will influence various attractive and repulsive forces. The extraction procedure has to be so designed as to exploit the differences in the physical properties of the analyte and the unwanted components of the sample.

Often it is useful to start from a point where information about separating other substances, having similar chemical structure, is available. McDowall *et al.* [1], in their guidelines for selecting a suitable SPE column, have pointed out that the important aspects to be considered are: literature search for a method for extracting a structurally related compound, consideration of physico-chemical properties of analyte and sample matrix and information on the compound's HPLC mobile phase. Although the permutations and combinations of the different types of sorbent and elution solvent are virtually endless, the SPE technique provides the analyst an opportunity to tailor-make the extraction procedure suitable for the particular analyte of interest.

Generally, to extract a non-polar compound from a polar biological sample, such as urine or plasma, a non-polar column such as, octadecyl (C_{18}) is chosen. The compound is retained by the sorbent and the unwanted polar constituents pass through the column to waste. The compound is then eluted from the sorbent using a non-polar solvent, such as chloroform.

C_{18}-bonded silica columns have been used very widely for extracting compounds from different biological sample fluids. Hartley *et al.* [13] have compared the extraction efficiency of Bond Elut® (Analytichem International) C_1, C_2, C_4, C_6, C_8 and C_{18} columns, using chlormethiazole edisylate and nitrazepam as model analytes. According to these authors the percentage of carbon loading is an important factor in determing the recovery, and the carbon number is less important. The Bond Elut® columns had a carbon loading percentage between 4.5 and 18. Furthermore, they compared the results with SepPak® (Waters Associates) C_{18} columns having carbon loading percentage between 10 and 11. The higher recoveries were obtained with Bond Elut® C_6, C_4 and SepPak® C_{18} columns, all of which had a carbon loading percentage between 8.5 and 11. However, the Bond Elut® C_{18} column with 18% carbon loading gave poorer results.

According to Musch and Massart [14] a CN-bonded silica column, due to its intermediate polarity, permits the isolation of compounds from both polar as well as apolar media. Thus a CN-bonded phase permits a strategy to be developed for extracting most compounds. They screened about 30 basic compounds and found that those with a chain length of 11 or more carbon atoms got 99–100% adsorbed by the CN column. The column could be washed with water to remove polar contaminants. The compounds could then be efficiently recovered using methanol or acetonitrile–buffer (pH 3) as eluents. The authors explained that the interactions of CN sorbent in a polar environment are due to both the hydrophobicity as well as the polar characters of silica and residual silanols. In another paper, Musch *et al.* [15] have developed a general strategy to extract 13 different β-blockers from plasma samples, using CN-bonded columns. Deproteinized plasma samples were added to CN-bonded columns which were preconditioned with methanol and water. After removing the polar impurities, the β-blockers were extracted with acetonitrile–phosphate buffer (pH 3). The percentage recoveries were between 79 and 99.

Schwende *et al.* [16] have reported the suitability of carboxylic acid derivatized silica columns for extracting alentamol hydrochlor-

ide from human serum and urine. They have indicated the suitability of this column for basic compounds.

The recently introduced copolymeric bonded silica columns, such as the Bond Elut Certify® (Varian sample preparation products), possess both lipophilic and ion exchange properties. According to Dumasia and Houghton [17] these columns provide a simple, efficient and selective method for extracting a broad range of basic drugs. They have used these columns to extract β-adrenergic drugs from horse plasma and urine samples. The same columns were used by Dixit *et al.* [18] to extract fluoxetine and norfluoxetine from serum samples. Chen *et al.* [19] have described a unique procedure to extract acidic, neutral and basic drugs using Bond Elut Certify® columns. Calf plasma, spiked with methamphetamine, hexobarbital, mepivacaine, trimipramine, levellorphan and nitrazepam, was first diluted with phosphate buffer (pH 6) and then loaded to Certify® columns. Methylene chloride was used to elute the acidic and the neutral drugs and ethylacetate was employed to elute the basic ones. The mean percentage recoveries varied between 86 and 112. These copolymeric-bonded silica sorbents, depending on the elution fluid and pH condition, can have three different types of interactions: hydrophobic, polar and ionic. These interactions provide the selectivity, resulting in extremely clean extracts [17, 18].

For analytes, with ionizable functionality, ion exchange sorbents would be more suitable. Edelbroek *et al.* [20] have reported the use of quaternary amine columns for the selective extraction of the oral anticoagulant, phenprocoumon, from samples of untreated and β-glucuronidase or sulphatase treated urine. Urine samples were adjusted to pH 6.5–7, before loading them to the columns. The columns were then washed with 0.3 M ammonium formate, followed by water, to remove unwanted substances. The unconjugated and conjugated phenprocoumon were eluted with 0.1 M diammonium hydrogen citrate (pH 3.5)–methanol (7:3, v/v) and tetrahydrofuran, respectively. The authors have proposed that an ion-pair bond formation, between the amine group of the column sorbent material and oxygen anions of phenprocoumon, is responsible for its selective retention in the column.

Often it becomes necessary to use more than one column to achieve the desired sample purity. Suss *et al.* [21] have used, C_{18}-reversed-phase and benzene sulphonic acid-cation exchange, SPE columns in sequence to extract benperidol and its metabolites from human plasma. The use of two different types of columns enabled them to use different solvents, such as water, methanol and 40% methanol–water mixture, to wash-out polar and non-polar interfering substances and achieve a minimum detectable sensitivity of 0.5 ng ml^{-1} of benperidol from plasma. Although Kiang and Lee [22] could extract 1,4-diketolonapalene from rat urine using a C_{18} column alone, they had to pass the urine sample through both phenyl and silica columns, in sequence, to obtain 1,4-diketo 2,3-dihydroxylonapalene. The authors have explained that for selective retention of the polar dihydroxy metabolite, it is necessary to have a polar adsorbing surface. Some of the other examples of multiple column use are extraction of dopamine sulphate isomers from human urine using C_{18} and anion exchange SPE columns in sequence [23], extraction of homovanillic acid from human plasma using C_8 and anion exchange columns [24], and extraction of steroids from human and calf urine using NH_2 and C_{18} columns [25, 26].

Many workers have observed differences in percentage recovery while using the same type of columns supplied by manufacturers [20, 27–29]. Raune and Wilson [28] have reported differences in the recoveries among the C_{18} columns supplied by Analytichem International and J.T. Baker and have attributed the difference to the varying degree of coverage with C_{18} groups and the extent of end-capping of residual silanols. It is advisable to use columns having the same lot number for one set of experiments [20, 29]. Manufacturers have now addressed this issue by packing columns having the same lot number in one box.

Types of Sample

The SPE technique has been employed to extract different kinds of samples. Those of biological origin, belonging to both humans as well as animals, are whole blood [30], plasma [31], serum [32], urine [33], faeces [34], tissue [35], vitreous humour [36], seminal fluid [37], saliva [38] and bile [39]. The non-biological

samples include beverages [40], milk [41], fruits [42], river water [43], soil [44], etc.

Advantages

SPE is a unique technique having many advantages, which is making it increasingly popular. It has revolutionized the sample preparation procedure due to its efficiency with regard to recovery, labour, solvent consumption and feasibility for complete automation of analysis.

The most significant merit of SPE, is the higher recovery with a high reproducibility, as compared with LLE. The percentage recoveries, reported in the literature using the SPE technique, range from 80 to as high as 100 [13, 28, 45, 46]. Sandberg [45] has compared the SPE and the LLE techniques for extracting four compounds. The percentage recoveries for lidocaine, cocaine, benzoylecgonine and norcocaine were 92, 86, 91 and 85, respectively, using a SPE procedure, as compared with only 59, 57, 66 and 21, respectively, using a LLE method. The RSDs were less than 7% for the SPE results, whereas for the LLE they were between 9.7 and 16%. Okazaki *et al.* [47] in their comparative study for the extraction of oflaxacin and its dimethyl and *N*-oxide metabolites have reported recoveries of 39.5, 54.7 and <5%, respectively, for the three analytes using LLE as compared with 98% recovery for all analytes using C_8 SPE columns. Wientjes *et al.* [48] have also compared the two techniques while extracting 2′-3′-dideoxyinosine from human plasma and recovered only 33% using a LLE method as compared with more than 90% recovery while using C_{18} columns in a SPE procedure. The other studies comparing the two techniques have also favoured SPE over LLE [49–51].

High recovery of an analyte makes an assay procedure more sensitive and this, in turn, enables a smaller sample size to be quantified successfully. Hoke *et al.* [43] have used C_{18} SPE columns to pre-concentrate mycotoxin from river water thereby increasing the sensitivity of artemia bioassay, by more than a factor of five. Wells *et al.* [52] have reported a SPE method to extract 0.2 ml of plasma and urine samples, taken from human neonates, to study the pharmacokinetics of bumetanide. Such a study would not have been possible earlier, since the previous conventional method needed a larger sample making it unsuitable for clinical studies on neonates and infants. Sioufi *et al.* [53] have used a SPE technique to extract minute quantities of nicotine from human samples after treatment with transdermal patches of nicotine, achieving detection sensitivities of 1 and 20 ng ml^{-1} in plasma and urine, respectively. March *et al.* [54] extracted torsemide, a potent diuretic given in very small doses, using a SPE procedure and detected 10 and 20 ng ml^{-1} of plasma and urine, respectively. Huang *et al.* [55] succeeded in analysing plasma sample, as small as 0.2 ml, to quantify etintidine, using C_8 SPE column.

Another significant advantage of using the SPE technique is its efficiency with regard to economy of time and labour. The commercially available extraction manifolds can handle up to 24 samples simultaneously and the entire procedure takes much less time compared with a LLE procedure. Forrest *et al.* [12] have reported taking <5 min to extract 10 samples. By employing such a rapid procedure it is now possible to treat samples containing volatile analytes such as, chlormethiazole [13] and 3-methylindole [34]. Wise [56] has successfully extracted retinoids, intact, which are otherwise sensitive to oxidation and isomerization, from human samples using a SPE procedure. Nitsche *et al.* [57] employed an on-line SPE assembly attached to a HPLC unit to analyse the photolabile compound, nifedipine, from plasma samples and thus avoided its decomposition by light.

Besides the economy of time and labour, the SPE technique provides considerable savings in the amount of organic solvents used for elution. Generally, only few millilitres of solvents are needed for the extraction procedure. Reduction in the amount of organic solvent helps in saving costs, minimizes the problem of waste-disposal thus making it more environment-friendly, reduces potential fire hazard and promotes safer working conditions for the laboratory personnel. Kupferschmidt *et al.* [58], adopted a SPE method to extract 3′-azido-3′-deoxythymidine from blood samples of AIDS patients since, besides providing other advantages, the method minimized sample handling, thereby, making it a safer laboratory procedure.

Generally, SPE columns are meant for single use and the manufacturers do not recommend repeated use. However, the analyst may explore the possibility of reusing the SPE

columns which will depend on the type of column, sample and solvents used. In many instances, it may be possible to develop a suitable method for regenerating column sorbents. In the literature, there are several reports of repeated use of the disposable columns [41, 59–61]. This could result in further savings in analytical cost. Van de Water *et al.* [41] have reported using carbonyldiimidazole activated support, more than 30 times without any significant reduction in column capacity. Milne *et al.* [62] have used C_{18} SPE columns repeatedly to treat 14 replicate plasma samples containing spiked morphine and its metabolites. The percentage recoveries obtained were within ±5 SD and they observed no particular trend in the quantitative values and the HPLC peak profiles.

Another useful feature of the SPE technique is its versatility in being able to handle different types of samples. The biological samples may be subjected directly to a SPE procedure without any prior treatment thus simplifying the extraction procedure. Moor *et al.* [63] have reported the use of C_8 columns, to extract amiodarone and desmethyl-amiodarone from plasma and different tissues of rat and found that the efficiency of extraction procedure was independent of the amount and kind of biological sample. Hattori *et al.* [64] employed C_{18} columns to extract local anaesthetics, from whole blood and cerebrospinal fluid samples, without any prior treatment. The samples were diluted nine times with water before passing them through SPE columns. The columns were then washed with 10 ml of water prior to elution of the anlytes with a chloroform–methanol mixture (9:1, v/v). According to Goto *et al.* [65], the commonly employed method, involving prior solvolysis and/or hydrolysis step, in the extraction of 3-oxobile acid from serum, lacks reliability and does not provide information about the conjugated forms. Moreover, 3-oxobile acid, being unstable in alkaline pH, is also susceptible to degradation. By using a SPE procedure, which involved no prior treatment, they could avoid these problems and improve the analyte's recovery. Stewart *et al.* [66] found that the recovery of chloroxazone and its hydroxy derivative from plasma improved from 22.6%, obtained with a protein denaturation step prior to SPE, to 90.2% without any denaturation step. However, Bauerova *et al.* [67] have reported a study, exemplifying the counter point, in which skipping the plasma protein precipitation step prior to the SPE step led to 50% loss of pentoxifylline. Thus it may be difficult to generalize the procedure with regard to pretreatment and the analyst has to determine the best conditions suitable to a particular sample.

Applications

The conventional LLE can be replaced by the more efficient SPE to treat a wide variety of biological and non-biological samples. Besides the general extraction application, SPE can be applied in several special instances.

Svensson *et al.* [68] have used SPE as a time and cost saving technique for routine clinical analysis of thioridazine and found it very convenient to analyse a large number of samples. Dalbacke *et al.* [69] have reported aspirating 1 l of drinking water, containing 0.1 mg of warfarin, through a C_{18} SPE column and eluting the drug with 1 ml of solvent. McCormick [70] has used a SPE procedure to remove tens of milligrams of proteins from sub-microgram quantities of nucleic acid and quantitatively recovered it in biologically active state. They found the SPE method advantageous with regard to speed, high recovery, safety and convenience.

SPE has found its application in law enforcement laboratories as well where contraband substances, present in trace amounts in different biological samples, have been quantified. Several papers [71–77] presented at the Second International Symposium on Analysis of Anabolizing and Doping Agents in Biosamples, held in Ghent, Belgium in May 1990, on the analysis of anabolizing and doping agents, such as testosterone, progesterone, ethinylestradiol, diethylstibesterol and trenbolone, etc. in biosamples, employed SPE technique for extraction. The high efficiency of the SPE technique can help in detecting minute quantities of anobolic steroids and doping agents which are forbidden from being used in feeding live stocks catering the meat industry.

Some of the analytical instruments, such as gas chromatography–mass spectrometry (GC–MS), require very high sample purity. A SPE procedure can provide the required purity along with high recovery. Brownshill *et al.* [78] have developed a procedure involving multiple SPE steps to obtain fenfluramine from human urine, having a purity suitable for GC–MS

study. In this method the urine sample was passed, in sequence, through XAD-2 resin and C_8 columns before subjecting it to semi-preparative HPLC gradient elution. After enzymatically hydrolysing the conjugate metabolites, the extract was further purified using a C_8 cartridge before finally derivatizing them for a GCMS analysis.

J.T. Baker Inc. has introduced SPE disks to analyse large-volume samples, such as those from environmental studies. These are chemically bonded silica enmeshed in inert PTFE matrix. These disks, available in 25 and 47 mm diameters, provide a larger surface area, for handling high volumes of samples, without sacrificing any of the advantages of the SPE technique. Evans *et al.* [79] have used such disks to extract tributylin from large volumes of marine samples.

The SPE technique may be used either separately, off-line, to prepare cleaner samples prior to analysis or, as an on-line trace enrichment technique where, by column-switching device, the final eluent from the SPE column is injected to a HPLC system. Both methods have their own merits.

The on-line technique involves critical issues such as, length of pre-column, straight or back-flushing, optimization of pre-column coupling time, etc. all of which need not be worried about in off-line [14]. The on-line trace-enrichment technique is often time-consuming to operate manually and direct injection of sample causes frequent blocking of both trace-enrichment and analytical columns [12]. This method may also lack flexibility and unless a sophisticated column-switching system is used, it is limited to a simple wash step, followed by elution with HPLC mobile phase, whereas in an off-line method a variety of solvents and multiple columns can also be used to suit ones needs [1]. However, the on-line technique has been used by many workers since it can be easily automated and, once perfected, is rapid, precise and cost-effective [55, 80]. The on-line extraction process is generally linked to a HPLC quantification system through a column-switching device. Such a system is ideally suited for analysing a photosensitive compound, such as nifedipine [57]. Neubert *et al.* [80] adapted the SPE technique to efficiently extract adibenden and its metabolites from dog urine, using a multiple-injection device, resulting in extraction from several successive traces on the pre-column.

With the introduction of the SPE technique it is now possible to completely automate an analytical procedure. Depending on the choice of the system, automation could be either semi or complete. The semi-automatic module is microprocessor controlled. It is manual up to the point of removing the interfering substances from the SPE column. The columns, usually 10 of them grouped in a cassette, are loaded on to an automatic sample preparation unit, which does the rest from the elution of the analyte onwards.

Complete automation involves robotics and is more expensive. However, in industries, where large numbers of samples are handled and analytical precision with speed is of prime importance, robotics have found greater utility. A robotic system, where SPE is an integral part, needs no supervision and about 100 samples can be easily analysed in a day [81, 82].

Conclusion

The SPE technique has revolutionized sample preparation procedure and provides many advantages as compared with conventional LLE. It can be suitably adapted to ones needs, to extract analytes from almost any kind of sample, with a high percentage of recovery and purity. SPE can be used either manually or attached to more sophisticated systems to achieve semi or complete automation of sample analysis.

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