

3 Sample Preparation

There has been unprecedented growth in measurement techniques over the last few decades. Instrumentations, such as chromatography, spectroscopy and microscopy, as well as sensors and microdevices, have undergone phenomenal developments. Despite the sophisticated arsenal of analytical tools, complete noninvasive measurements are still not possible in most cases. More often than not, one or more pretreatment steps are necessary. These are referred to as sample preparation, whose goal is enrichment, clean up, and signal enhancement. Sample preparation is often the bottleneck in a measurement process, as they tend to be slow and labor-intensive. Despite this reality, it did not receive much attention until quite recently. However, the last two decades have seen rapid evolution and an explosive growth of this industry. This was particularly driven by the needs of the environmental and pharmaceutical industries, which analyze large number of samples requiring significant efforts in sample preparation [66]. Some common steps involved in the process analysis including sample preparation steps are shown in Figure 3.1.

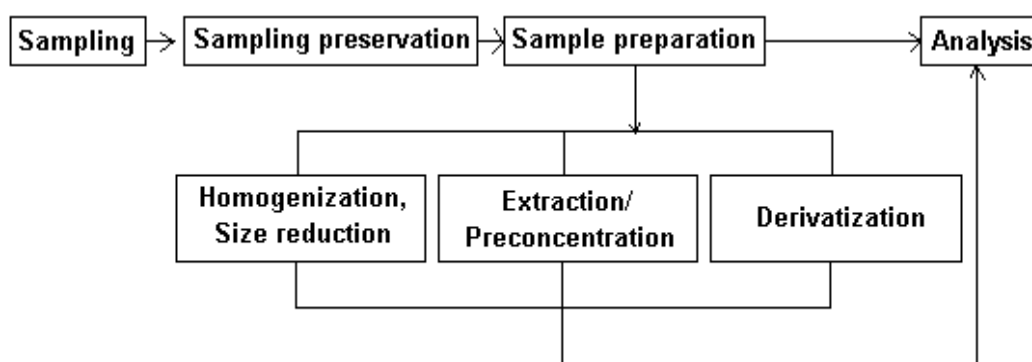
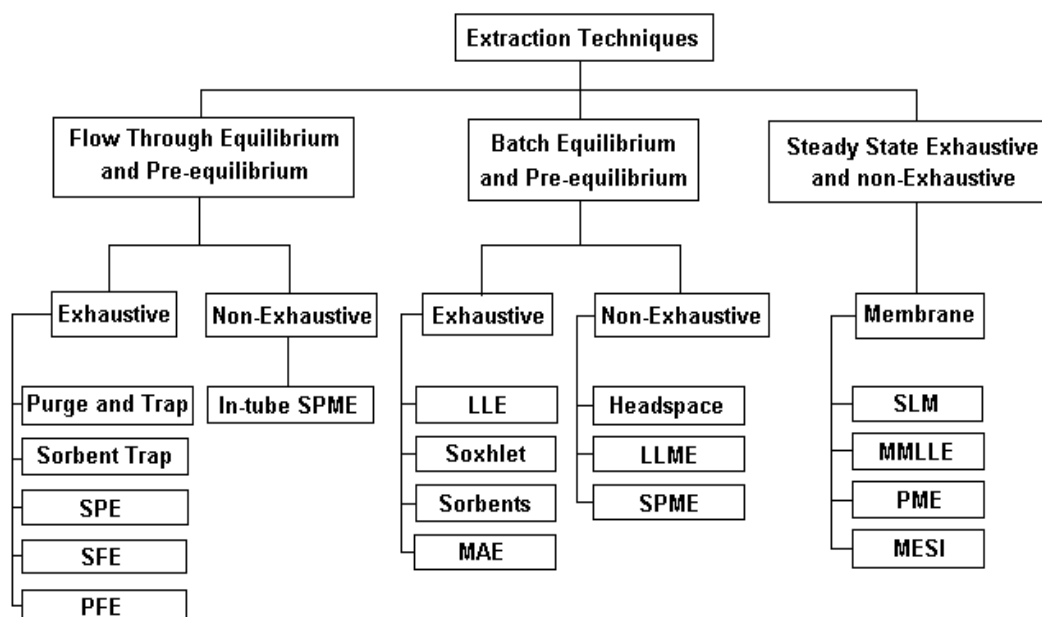


Figure 3.1 Steps in a process analysis including sample preparation

3.1 Extraction

The sample preparation step in an analytical process typically consists of an extraction procedure that results in the isolation and enrichment of components of interest from a sample matrix. Extraction can vary in degree of selectivity, speed, and convenience and depends not only on the approach and conditions used but on the geometric configurations of the extraction phase. Increased interest in sample preparation research has been generated by the introduction of nontraditional extraction technologies. These technologies address the need for reduction of solvent use, automation, and miniaturization and ultimately lead to on-site in situ and in vivo implementation. These extraction approaches are frequently easier to operate but provide optimization challenges. Optimization of this extraction process enhances overall analysis. Proper design of the extraction devices and procedures facilitates convenient on-site implementation, integration, integration with sampling, and separation/quantification, automation, or both.

A classification of extraction techniques and unifies the fundamental principles behind the different extraction approaches is shown in Figure 3.2.



SPE = Solid Phase Extraction; SFE = Supercritical Fluid Extraction; PFE = Pressurized Fluid Extraction; SPME = Solid Phase Microextraction; LLE = Liquid Liquid Extraction; MAE = Microwave Assisted Extraction; LLME = Liquid Liquid Microextraction; SLM = Supported Liquid Membrane extraction; MMLLE = Microporous Membrane Liquid-Liquid Extraction; PME = Polymeric Membrane Extraction; MESI = Membrane Extraction with Sorbent Interface

Figure 3.2 Classification of extraction techniques

3.1.1 Liquid-liquid Extraction

Liquid-liquid extraction (LLE), or solvent extraction, is one of the oldest and most widely used techniques in the preparation of samples for qualitative and quantitative analysis. It involves the distribution of the sample components between two immiscible liquid phases. As for any sample preparation technique, sample clean up and/or analyte preconcentration are the most common goals of LLE. The first requires a high selectivity for partitioning of the analyte component over that of potential interference; while the second is favored by a high distribution ratio, so that the analyte component can be extracted from a large volume of sample solution into a small volume of extractant.

In its most classic form it is performed in a separatory funnel by agitating the mixture to disperse drops of one liquid in the other, discontinuing agitation to allow drop coalescence, and then separating the bulk liquid phases from one another. This mode of operation introduces some practical problems because it is not possible to work with phase ratios (V_o/V_a) smaller than 0.01, which militates against a high preconcentration ratio [67]. In addition, coalescence and phase separation can be slow, especially if emulsions are formed [68]. This is a particular problem for samples of natural origin, such as urine and vegetable matter extracts, which contain surface-active agents that adsorb at liquid-liquid interface to produce emulsions. Another disadvantage is the relatively large amount of waste organic solvent that is generated. Therefore, because of practical problems associated with the separatory funnel mode of operation, LLE has been significantly replaced in recent decades by other type of batch-phase distribution techniques, such as solid phase extraction (SPE) and solid phase microextraction (SPME). However [67], LLE offers large linear sample capacities compared to other batch-type phase-distribution techniques. This is because its full-phase volume is available so that competition for space in the extract phase is minimal. The second advantage is the fact that the organic extract itself can be directly subjected to the quantitative analytical measurement step such as gas or liquid chromatography. In other hand, back extraction, in which the analyte component is removed from the organic extract phase, can intentionally be used to add extra selectivity or an extra degree of preconcentration of the analyte component and, furthermore, back extraction can be performed simultaneously with forward extraction. A final advantage of LLE derives from the vast body of solvent extraction literature, accumulated over many decades, which provides information on choice of organic solvent, of pH and of type and concentration of reagents.

More recently, a variety of non classical modes of performing LLE have been described which circumvent the practical problems and beg a critical re-evaluation of the role of LLE in sample preparation. These non classical modes of LLE are [67] segmented flow techniques, single liquid drop techniques, unsupported liquid membrane techniques with three phases, and supported liquid membrane techniques. The last three techniques mentioned above, taken as a group, are sometimes referred to as liquid phase microextraction (LPME) techniques.

Segmented flow techniques in small bore tubes performed in the flow injection analysis mode, were initially developed in the late 1970s and early 1980s. Continuous, co current flow of alternating aqueous and organic solvent segments occurs, either with or without air bubble segmentation. Mechanical phase separators sort the organic from the aqueous segments at the outlet end of the extraction tube, or on-tube detection is used.

Single liquid drop techniques with two phases employ a microliter or smaller size drop of organic solvent phase suspended in a large volume of aqueous sample phase. When extraction is terminated, the drop can be injected directly into an instrument such as chromatograph or a graphite furnace atomic spectrophotometer, or it can be interrogated in situ by absorption or fluorescence spectroscopy.

Unsupported liquid membrane techniques with three phases involve an aqueous sample phase separated from an aqueous receiver phase by a layer of organic solvent. The conditions in the aqueous sample phase so that the analyte is forward-extracted out of the sample phase into the organic phase and back-extracted out of the organic phase into the receiver phase, all in one simultaneous operation.

Supported liquid membrane techniques employ either two or three phases, with simultaneous forward- and back-extraction in the latter configuration. The aqueous sample phase is separated from the bulk organic or from an aqueous receiver phase by a porous, polymer membrane, in the form of either a flat sheet or a hollow fiber that has been impregnated with the organic solvent phase. The sample phase is continuously pumped, the receiver phase may be stagnant or pumped, and the organic phase in the membrane pore is stagnant and not replaced between samples.

3.1.2 Liquid-solid Extraction

Liquid solid extraction has been common placed in flow systems since the pioneering works employing ion exchange resins [69] through the more recent contributions, which describe the use of reactive solid phases and in situ monitoring of the absorbed species [70-72]. Flow injection analysis has been the preferable flow technique employed to mechanize the liquid-solid extraction because, in most cases, the solid phase is retained in a small column inserted on the way to the detector. The literature reports on preconcentration gains more than 40 in many cases. However, the inherent dispersion caused during analyte elution from the solid phase in a non-segmented flow system reduces the gain for preconcentration.

The use of liquid-solid extraction batch procedure where the entire extracting solid phase is, after preconcentration, dissolved in a suitable solvent (usually an organic one) in order to recover the absorbed analyte for detection, is also employed. In this process, the solid phase is prepared to contain a suitable ligand, which reacts with the analyte and simultaneously retains the complex in the solid matrix. The whole solid phase is dissolved in an organic solvent, and transferred to a volumetric

flask, diluted and presented to various detection techniques. Obviously, this procedure employs a new organic phase for each extraction and reversibility of the absorption process is not required. On the other hand, this aspect imposes some difficulties for the mechanization of the procedure by, for example, a flow analysis approach. The apparent difficulty is also renew the solid phase between successive extractions. The batch procedure aims at 100% absorption and recovery. The preconcentration gain is obtained by the ratio between the volume of the aqueous phase from which the analyte is absorbed and the volume of organic phase employed to dissolve the solid phase after extraction. Typical gains are in the range of 20-100.

Neto and Pasquini [73] developed a new flow analysis approach to mechanize the liquid-solid extraction procedure using a renewable extracting solid phase with recovery of the organic phase after dissolution and spectrophotometric detection of the extracted analyte. The development and evaluation of the characteristics of the system has been made by employing Zn(II) as analyte and 1-(2-pyridylazo)-2-naphthol (PAN) incorporated in naphthalene as the extracting solid phase.

3.1.3 Solid-phase Extraction

Classical extraction procedures consume large amounts of solvents, thus themselves creating environmental and occupational hazards, and often provide very little selectivity. The analytical community responded to this challenge by increasing research on sorbent traps, solid-phase extraction (SPE), solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE) as other, less-solvent-consuming, alternatives to charcoal tube, liquid-liquid, and Soxhlet extraction.

Solid-phase extraction refers to the nonequilibrium, exhaustive removal of chemical constituents from a flowing liquid sample via retention on a contained solid sorbent and subsequent recovery of selected constituents by elution from the sorbent [74]. Generally, SPE consists of four steps: column preparation (prewash), sample loading (retention or sorption), column postwash, and sample desorption (elution or desorption). The prewash step is used to condition the stationary phase if necessary, and the optional column postwash is used to remove undesirable contaminants. Usually, the compounds of interest are retained on the sorbent while interferences are washed away. Analytes are recovered via an elution solvent.

Appropriate SPE sorbent selection is critical to obtaining efficient SPE recovery of analyte from liquids. Henry [75] notes that an SPE sorbent must be able to sorb rapidly and reproducibly, defined quantities of sample components of interest. Fritz [76] states that successful SPE has two major requirements: (1) a high reproducible percentage of the analytical solutes must be taken up by the solid extractant; and (2) the solutes must then be easily and completely eluted from the solid particles. The sorption process must be reversible. In addition to reversible sorption, SPE sorbents should be porous with large surface areas, be free of leachable impurities, exhibit stability toward the sample matrix and the elution solvents, and have good surface contact with the sample solution.

3.1.4 Solid-phase Microextraction

Solid-phase microextraction (SPME) was introduced by Arthur and Pawliszyn [77]. By SPME, samples are analyzed after equilibrium is reached or at a specified time prior to achieving equilibrium. Therefore,

SPME operationally encompasses nonexhaustive, equilibrium and preequilibrium, batch and flow-through microextraction techniques. Although various ways to implement SPME are proposed and are being developed, there are two primary approaches to conducting SPME: with the sorbent coated on the outer surface of fibers or with the sorbent coated on the internal surface of the capillary tube. The fiber design can be interfaced with either GC or HPLC. However, the in-tube design has been developed as an easier approach for interfacing SPME with HPLC.

SPME can be conducted as a direct extraction in which the coated fiber is immersed in the aqueous sample; in a headspace configuration for sampling air or the volatiles from the headspace above an aqueous sample in a vial; or by membrane protection approach, which protects the fiber coating, for analysis of analytes in very polluted samples. The SPME process consists of two steps: (a) the sorbent, either an externally coated fiber or an internally coated tube, is exposed to the sample for a specified period of time; (b) the sorbent is transferred to a device that interfaces with an analytical instrument for thermal desorption using GC or for solvent desorption when using HPLC.

3.1.5 Stir Bar Sorptive Extraction

Stir bar sorptive extraction (SBSE) was recently introduced [74] for the trace enrichment of organic compounds from aqueous food, biological, and environmental samples. A stir bar is coated with a sorbent and immersed in the sample to extract the analyte from solution. The sample is typically stirred with the coated stir bar for a specified time depending on the sample volume and the stirring speed, to approach equilibrium. The sorbent used for coating the stir bar is polydimethylsiloxane (PDMS).

Using this sorbent, the primary mechanism of interaction with organic solutes is via absorption or partitioning into the PDMS coating. Extraction of aqueous samples occurs during stirring at a specified speed for a predefined time. After a given stirring time, the bar is removed from the sample and is usually thermally desorbed into a gas chromatograph.

However, there is a fundamental similarity among the extraction techniques used in the sample preparation process. In all techniques, the extraction phase is in contact with the sample matrix and analytes are transported between the phases.

3.1.6 Extraction and Preconcentration in the FIA of Pharmaceuticals

Single extraction without phase separation has been used in the determination of benzethonium and berberine chlorides in disinfecting medicines after the extraction of the colored product of their reaction with Bromochlorophenol and Quinidine using dichloroethane by injecting the reactant into an extractant flow, which was continuously pumped through the extraction coil to detector [78]. An extraction system was proposed for the spectrophotometric-FIA determination of Salbumol after its oxidative coupling with 4-amino-N,N'-dimethylaniline [79]. In number of cases, online single extraction with phase separation was used in FIA. Such an approach was used in the determination of Bi(III) in pharmaceutical preparations as an ion associate of tetraiodobismuthate in a segmented-flow mode (20 samples/h, detection limit $0.24 \mu\text{g mL}^{-1}$ for a sample volume of $250 \mu\text{L}$) [80]. Berberin in oriental medicine preparations was determined as ion associate with perchlorate using fluorescence detector with detection limit of $8 \times 10^{-10} \text{ M}$ (40 samples/h). Fujiwara et. al. [81] devised instrumentation for online, continuous ion-pair formation and solvent

extraction, phase separation, and detection. The procedure was applied to the determination of atropine in synthetic urine, and of atropine and scopolamine in standard pharmaceuticals.

Multiple extractions were also used in order to increase the selectivity and sensitivity. Multiple extractions of ion pairs of the pharmaceutical with ethyl ether of tetrabromophenolphthalein were used in determination of cetylpyridinium chloride in medicinal preparations [82]. Similar extraction-based systems were used for the flow injection determination of caffeine [83], codeine [84], and steroids [85].

Coupling of FIA with dialysis system, especially for in-vivo determinations, has also been reported. A flow injection determination of L-amino acids in mixtures with other amino acids using electro dialysis was described in [86]. Hydrogen peroxide formed after the separation of amino acids and performing enzymatic reactions was determined electrochemically. Dialysis systems were used in FIA both for the analysis of proteins [87] and for their separation in the determination of glucose, urea, galactose, and lactose [88-90]. These systems provide continuous online monitoring of glucose in blood [91]. The determination of pharmaceuticals in the dialyzate was made after an enzymatic reaction with glucose oxidase using the chemiluminescence detection of the formed hydrogen peroxide after its reaction with luminol.

3.2 Derivatization

The specific properties of the vast majority of substances in pharmaceutical and their metabolites have high polarity and weak chromophoric, electrophoric, or fluorophoric properties and often restrict

the possibility of their selective determination. Moreover, measuring analytical signals under non-equilibrium conditions (when physical and chemical processes in the reactor are not completed) requires the high selectivity and sensitivity of the substances. Derivatization is still the major approach to improve the analytical properties of the substance that aims at the synthesis of new compounds with better characteristics compared to the original substances. Derivatization also improves the extraction properties of analytes, enhances their stability in the course of analysis, simplifies and facilitates sample preparation, and decreases the limits of detection.

Various chemical, photochemical, and enzymatic reaction were used for producing derivatives. Reactions could take place during the movement of the reaction zone to the detector or directly in the detector during measurements. In many cases, reactions used for the derivatization of pharmaceuticals are slow, possess complex mechanisms, and depend on the composition of the solution, pH, and other factors. The chemical reactions and reagents used for the derivatization of pharmaceutical are presented in Table 3.1.

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Table 3.1 Chemical reactions and reagents used for the derivatization of pharmaceuticals [25]

Type of derivatization reaction	Reagents	Classes of determined pharmaceuticals	Main detection methods
Formation of azo compounds	Sodium nitrite and amines	Sulfanilamides	Spectrophotometric
Condensation with carbonyl compounds	<i>o</i> -Phthalic aldehyde and 2-mercaptoethanol, <i>p</i> -dimethyl-aminobenzaldehyde	Sulfanilamides, amines, and alkaloids	Fluorimetric
Redox processes	Metal ions, oxidant anions, hydrogen peroxide, oxidases, reductases	Phenothiazines, catecholamines, antibiotics, amino acids, ureas, steroids, vitamins, and organic acids	Spectrophotometric, fluorimetric, and electrochemical
Complexation	Metal ions	Antibiotics, organic acids, and amines	Spectrophotometric and fluorimetric
Nucleophilic substitution	7-chloro-4-nitrobenzofurazan, 4-chloro-5,7-dinitrobenzofurazan, 7-chloro-4,6-dinitrobenzofuroxan, 1-fluoro-2,4-dinitrobenzene	Arylamines, hydrazides, aminophenols, sulfanilamides, heterocyclic amines, phenol derivatives, and catecholamines	Spectrophotometric, fluorimetric, electrochemical
Salt formation	Organic dyes, salts	Phenothiazine, organic salts, and amines	Spectrophotometric

Photochemical derivatization is a promising way to expanding the possibilities of flow injection analysis of pharmaceuticals. Photochemical derivatization gives rise to a variety of products of photochemical transformations of the compound to be determined and sharply increases in the intensity of the measured signal. The characteristics of procedures for the flow injection determination of pharmaceuticals with photochemical derivatization is presented in Table 3.2

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Table 3.2 The characteristics of procedures for the flow injection determination of pharmaceuticals with photochemical derivatization[25]

Pharmaceutical	Conditions of photochemical derivatization, detection method	Linear calibration range	Detection limit
Thiamine	Photodecomposition in acidic solution, UV photometry	1.2-30 µg/mL	
Acetylcholine, choline	Chemiluminescent reaction of luminol and H ₂ O ₂ in micellar solution	5 x 10 ⁻⁷ -10 ⁻⁴ M	5 x 10 ⁻⁸ M
Kanamycinum	Chemiluminescent reaction of luminol and H ₂ O ₂ in the presence of Co(II)	10 ⁻¹³ -10 ⁻⁵ M	
Epinephrine, norepinephrine, dopamine, L-dopa	Measurements of chemiluminescence in potassium permanganate-formaldehyde system	0.05-1.0 µg/mL	
Isoprenaline	Measurements of chemiluminescent reaction with lucigenin in alkaline solutions	10 ⁻⁷ -10 ⁻⁴ M	
Steroids	Measurements of chemiluminescence in Ce(IV)-sulfite system	0.02-1.00 µg/mL	0.013-0.019 mg/mL
Riboflavin	Photochemical reduction of EDTA followed with the reaction with H ₂ O ₂	10 ⁻⁷ -3 x 10 ⁻⁶ M	
Urea	Measurements of chemiluminescent reaction with hypobromite in alkaline solutions	5 x 10 ⁻⁷ -5 x 10 ⁻⁵ M	9 x 10 ⁻⁸ M
Chlorpromazine, promethazine	Photochemical derivatization, fluorimetry	0.5-8.0 µg/mL	
Ascorbic acid	Photooxidation in the presence of Toluidine Blue as sensitizer	1.1 x 10 ⁻⁹ -3 x 10 ⁻⁴ M	2 x 10 ⁻¹⁰ M
Ascorbic acid	Measurements of chemiluminescent reaction with lucigenin in micellar solutions catalyzed with Fe(III)	2 x 10 ⁻⁷ -6 x 10 ⁻⁷ M	2 x 10 ⁻⁹ M
Cephadroxy	Measurements of chemiluminescent in potassium permanganate-quinine in acidic solutions	0.1-30 µg/mL	0.05 µg/mL

In practice of FIA, manifolds for various homogeneous and heterogeneous reactions involving pharmaceuticals include reactor coils with immobilized reagents. Among them are spectrophotometric determinations of amino acids [92], promazine [93], and methotrexate [94] in medicinal preparations. In the first case, the reaction occurred on a polymer and led to the formation of colored complexes with zinc. In the other

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examples, oxidation took place in a column at the surface of Amberlite IRA-400 [93] or in a thermostated reactor in the determination of methotrexate [94]. In spectrophotometric flow injection determination of glutamic acid, the enzymatic reaction was conducted in a reactor with glutamate oxidase. The products of this reaction were converted to a quinonimine dye [95]. An enzymatic reaction in the flow system can also be used for exciting the fluorescence of derivatives. For example the bioluminescence determination of Mg(II) in blood serum and medicine [96]. Characteristics of flow injection procedures for determining physiologically active compounds using reactors with immobilized enzymes are presented in Table 3.3.

Table 3.3 Characteristics of flow injection procedures for determining physiologically active compounds using reactors with immobilized enzymes [25]

Analyte	Enzymatic reaction	Detection method	Calibration range
Penicillin	Transformation to penicillic acid in reactor with penicillinase	Electrochemical: amperometry	0.066-0.25 mM
Penicillin	Transformation to penicillic acid in reactor with penicillinase	Electrochemical: pH sensors	1-100 mM
Catechol, catechol-amines	Oxidation in the presence of ascorbic acid and tyrosinase	Electrochemical: amperometry	10^{-9} - 10^{-7} M
Bile acids, glucose, ATP	Reactors with hydroxysteroid dehydrogenase, glucose dehydrogenase, and glucose-phosphate dehydrogenase	Chemiluminescence	0.5-100 nmol
3-Hydroxybutyrate	Reactor with 3-hydroxybutyrate dehydrogenase and NaDH_2 -oxidase	Chemiluminescence	2×10^{-7} - 5×10^{-4} M
3-Hydroxybutyrate	Reactor with 3-hydroxybutyrate dehydrogenase	Fluorimetry	0.7-500 μM
Glutamic acid	Reactor with glutamate dehydrogenase	Fluorimetry	0.5-200 μM
L-dopa and carbidopa	Transformation to dopachromes using an extract from yam roots	Spectrophotometry	2×10^{-5} M
L-arginine	Transformation to ornithine and urea by the action of arginase	Spectrophotometry	0.038-7.5 mM
L-asparagine	Transformation of aspartates and ammonia by the action of asparaginase	Spectrophotometry	0.06-15 mM
Theophylline	Inhibiting action on alkaline phosphatase	Fluorimetry	0.1-200 μM