



## Alternative sample diluents in bioanalytical LC–MS

The problem of sample diluent in bioanalytical LC–MS is reviewed with a special focus on large-volume injections and non-miscible solvents with mobile phase components. These issues are related to the sample preparation approach, which in many instances provides the sample diluent before injecting this into the chromatographic column. The sample volume influences the quantitation limit of the chromatographic method, while its nature may influence the retention process of the injected analytes. The literature reports a few papers that are focused on alternative sample diluents in bioanalytical LC–MS that are generally non-miscible with mobile phase. The principle of this approach and some of its current bioanalytical applications from literature are discussed. However, more applications and more publications from HPLC users and vendors are expected in this field, which could prove its analytical importance and potential in bioanalysis.

LC–MS is nowadays the most utilized analytical technique in bioanalysis due to its sensitivity and better control of the selective determination of many species in biological matrices.

The current available LC with mass-spectrometric detection instrumentation, with low or high resolution, has become the method of choice in small-molecule drug bioanalysis (molecular mass less than 800 Da) and is also increasingly being applied as an alternative to ligand-binding assays for the bioanalytical determination of biopharmaceuticals [1]. The trend of eliminating 'old' detection approaches in HPLC techniques in favor of MS can easily be seen in the content of many papers from specialized journals such as *Journal of Chromatography B*, *Biomedical Chromatography*, *Bioanalysis* and *Journal of Pharmaceutical and Biomedical Analysis*. On the other hand, the introduction of a new generation of HPLC instruments (namely, UHPLC) that surpasses the pressure limitation of conventional equipment (400 bar) and that are capable of achieving LC separations at back-pressures up to approximately 1000 bar are now commercialized. This technology is based on small particle size (up to 2  $\mu\text{m}$ ) as the stationary phase material, which allows high-throughput methods for bioanalytical purposes to be developed [2].

However, the quality of analytical data is highly influenced not only by the analytical instrument used to analyze more or less number of samples, but also by the correctness and the approach of carrying out the **sample preparation** [3]. The main task of this step is to

make the samples of biological origin amenable to the HPLC–MS analysis, or to improve its analytical performances.

The importance of sample preparation methods as the first stage in any bioanalytical procedure is well known, and this also influences the performances of the chromatographic process and MS detection [3–5]. This step is mainly focused on the isolation, enrichment or structure modification of target analyte(s) using different types of LLE, SPE, solid-phase microextraction, matrix precipitation or derivatization. The final sample, before injection into the LC–MS system, can be discussed by analyte concentration factor, interferences retained into the sample, and sample solvent [6]. The solvent (diluent) of the final sample should exhibit a high solubility for target analytes, and be appropriate to both LC separation and MS detection [7,8]. It can also be discussed according to its miscibility with the mobile phase, and especially by its amount (volume) injected into the head of analytical column, which in turn influences the LOQ of chromatographic method [9,10]. The nature of stationary phase and column dimension are also important from this point of view. In some special cases, stability of target analytes is also an important issue related to the **sample diluent** and its injection into the column [11]. This Review emphasizes the importance of the injection diluent in LC–MS and new alternative solvents that can be used in this analysis, which are reported in literature. The injected sample amount and its solvent nature are two items that are discussed in this Review.

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**Key Term**

**Green solvents:** Solvents that have a minimum impact on environment.

**'Green' solvents used as sample diluents**

Organic solvents that can be considered as **'green' solvents** are desirable in practice because they avoid toxicity and environment problems [51,52], although there is serious debate on how green property can be measured [53]. They have rarely been used for analytical purposes due to the specific conditions allowed for non-miscible solvents: extraction yield, selectivity and cost. Among them, many of the ionic liquids are considered as green extraction solvents, although they are mostly used as synthesis solvents rather than solvents for analytical purposes [54]. A useful and green method for the extraction of fats and oils from biological matrices is Soxhlet extraction using d-limonene as the biosolvent, or green solvent, instead of n-hexane (petroleum solvent) [55]. Starting from this information, according to which limonene can be used as a green solvent, a study was recently focused on its potential in application to LLE-based separation of simvastatin, lovastatin, and their hydroxyacid metabolites from human plasma samples, followed by direct injection of samples [56]. In this study, it was shown that extraction of hydrophobic compounds from biological matrices with limonene is, in principle, possible, and high-volume injection of aliquots from this hydrophobic solvent can be directly injected into the chromatographic column. In this way, the problems related to the toxic character of the classic solvents and unpleasant working conditions for the analyst are overcome by this solvent, which is less volatile than lower hydrocarbon solvents. The possibility of 'greening' the sample preparation is not only a new trend in bioanalysis, but it is also necessary, mainly when large number of samples must be analyzed.

Substitution of acetonitrile as organic modifier in mobile phases for RPLC with mixtures of propylene carbonate and ethanol has been proposed as 'greener' for chromatographic applications. Of note, these additives in mobile phase proved not to significantly modify the chromatographic performance, such as the elution order, retention time, peak efficiency or symmetry, in several LC mechanisms such as RP, ion pairing and HILIC [57]. Moreover, the effect of this replacement of acetonitrile with mixture of propylene carbonate and ethanol seems not to influence the analyte detectability by UV-vis or MS. The advantages of replacing acetonitrile with propylene carbonate and ethanol mixture in RPLC for bioanalytical purposes has been shown in a recent study assaying enalapril and enalaprilat in human plasma [58].

**Conclusion**

Various sample diluents can be used in LC depending on the retention mechanism of the chromatographic process. They must solve the problem of solubility of analytes and not disturb the elution process. For this reason, whatever the solvent is, the injection volume must be small, up to 5–10 µl. For larger volume injection, the sample diluent must be in the mobile phase or a very similar solvent. To date, only a few alternative sample diluents are known for bioanalytical purposes. In RPLC some papers report that hydrophobic solvents can be successfully used in LLE, followed by high-volume injection of the organic phase. In this way, the tedious operations given by organic layer prelevation, solvent evaporation, and residue redissolution in a small volume of mobile phase as sample diluent are avoided. The utilization of such solvents in large-scale studies for bioequivalence purposes proved that they represent a very good solution for simplification of sample preparation, and we expect to see in the near future many studies from HPLC users and vendors relying on this approach, as well as new organic diluents being studied for sample injection. If they fulfill environmentally friendly conditions, this will be another huge advantage for bioanalysis and LC.

**Future perspective**

Due to the continuous demand for sample preparation to be simplified for bioanalysis, a focus on alternative diluents in LC–MS is expected to be of great interest. Direct injection of the organic phase resulting from LLE or SPE in the chromatographic column will improve some analytical parameters, such as time, precision and detection limit. There will be permanent interest in organic solvents that fulfill the conditions imposed by green chemistry. Due to its importance in separation of very polar compounds, HILIC will be extensively studied from the sample diluent point of view.

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