1
Introduction

When you as an analyst are facing the challenge of determining a certain substance in plasma, urine or tissue – it may be a drug, a metabolite of a drug or a substance belonging to an organism (= endogenous substance), then totally different approaches are possible. You could start with the question: "What has been published on this subject so far?" It can easily happen that a multitude of possibilities overwhelms you, especially with drugs that are frequently prescribed. As an alternative, you can contact friends and acquaintances among your colleagues, who have hopefully already had experience with that substance in order to get some hints. A third possibility would be an intellectual time out, which, however, often ends with a chaotic gathering of material and thus possibly creates more questions than it yields answers.

Have you not always looked for a tool to help you with such questions, with which you can approach a solution in due time and that quickly informs you whether your own instruments suffice to reach your aim? In this book you shall get to know an adequate approach. Parts of this concept are familiar to every analyst who is in clinical/pharmaceutical research. However, the radical nature of this approach that has proved its worth a thousand times with us and our work, makes it a means that you may like to apply again and again. The following three chapters each introduce a specific question and develop a solution for it. Please, apply at first your method to develop a solution and then compare, if the approach of this book is familiar to you and you are used to applying it anyway, or you perhaps get to know new tools in this book, which may help you to solve the questions quickly and well.

Plasma (serum) plays an important role in clinical practice or with bioanalytical tests performed by pharmaceutical laboratories. Occasionally, urine checks are also of great importance: when you observe and check the characteristics of an illness, when you supervise the therapy of an illness or when you prepare a mass balance of drugs (e. g., how much of an oral applied medication you find in the excrements of the patient). Whether the substance under study is the parent drug or is one or more of the numerous metabolites, is not important right now.

HPLC Methods for Clinical Pharmaceutical Analysis, First Edition. Hermann Mascher

1.1 First Question: Determination of Ibuprofen in Plasma

A colleague or a superior asks you to determine the concentration of an NSAR (nonsteroidal antirheumatic) in human plasma. Possibly the superior being a medical person says something like: "You have already analyzed substances in this field of therapy, thus you could determine this substance – couldn't you?" If you ask for further information, you possibly do not even get the name of the substance, but merely the name of the drug – the trade name – and perhaps the dosage. The superior, a clinical employee in our case, has administered Brufen and therefore needs – that is what you can see after you have had a short glance at the list of drugs – the determination of ibuprofen in human plasma. Now – how do you usually approach such a question? Do you look for a reference in technical literature? Do you call colleagues? Do you have a glance at the structural formula?

According to our experience we recommend to answer the following two questions first:

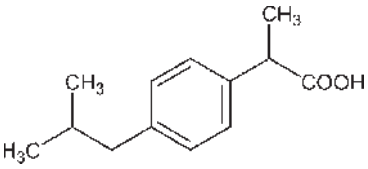
- Which determination limit is needed?
- Which kind of detector is at your disposal?

It is easy to answer the second question. For this example we suppose you have an HPLC instrument, a UV detector with variable wavelength and a fluorescence detector at your disposal. However, the question about the determination limit is much more difficult. What question does the superior want to solve? Does he need a check on the patient's compliance with the therapy? Is he concerned about too high plasma levels due to certain side effects? Or does he merely like to get to know minimal plasma concentrations before the next medication dosage ($C_{ss\ min}$)? For a maximum level at a usual medication dosage you find in the literature (e. g., "Martindale" is a good reference book[1]; or information given by the manufacturers of the drug) an approximate value of 20–50 µg/ml (see Appendix "Ibuprofen"). Also, details on elimination half-life are often important with such questions because they tell you when the clinical employee should draw the blood samples in order to answer his questions. Besides, the elimination half-life tells the analyst the plasma level he may expect, if – according to standard rules – blood samples are drawn approx. 5 h after the drug has been administered orally.

Back to the question: The superior in the given example is merely interested in the maximum level, because of the side effects. Thus, he must know that he has to carry out blood drawing after approx. 2 h and not right after oral administration or after 5 h even.

The analyst now knows that he has got to develop a safe proof within 10–60 µg/ml plasma. Actually, it looks like it would be simple: 10–60 ppm, whereas often proof is requested within the range of 10–60 ppb (10–60 ng/ml). At this point of development I recommend that you have a glance at the structural formula and at – as only UV and fluorescence detection are at you disposal – solubility as well as at the UV spectrum.

1 See list of books: Martindale



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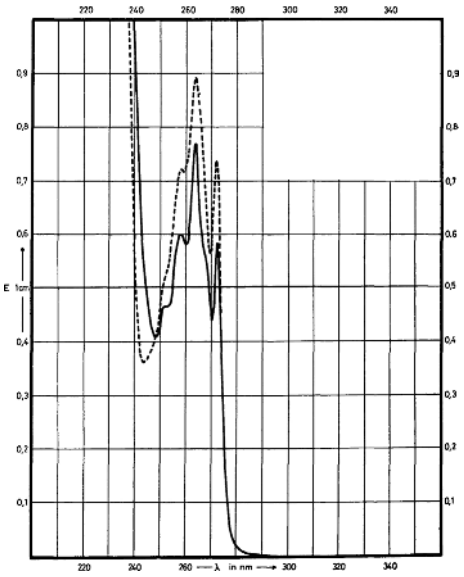


Table 1.1: Ibuprofen – Details to the UV-Spectrum

solvent symbol	**methanol** ———	**water** – · – · –	**0.1M HCl** – – – – –	**0.1M NaOH** · · · · · ·
absorption maximum	272 nm 264 nm 258 nm			272 nm 264 nm 258 nm
$E^{1\%}_{1cm}$	11.2 14.5 11.3			15.4 18.4 15.0
ε	230 300 233			320 380 310

- *Structure:* Ibuprofen tends to be a lipophilic substance, but it contains a carboxy function.
- Solubility: As a nondissociated substance in the acidic pH range it is lipophilic, as a salt (pH >6), however, presumably very well soluble in water.
- UV spectrum: Only below 230 nm do you get a reasonable absorption. Otherwise you cannot say whether the substance is fluorescent or not.

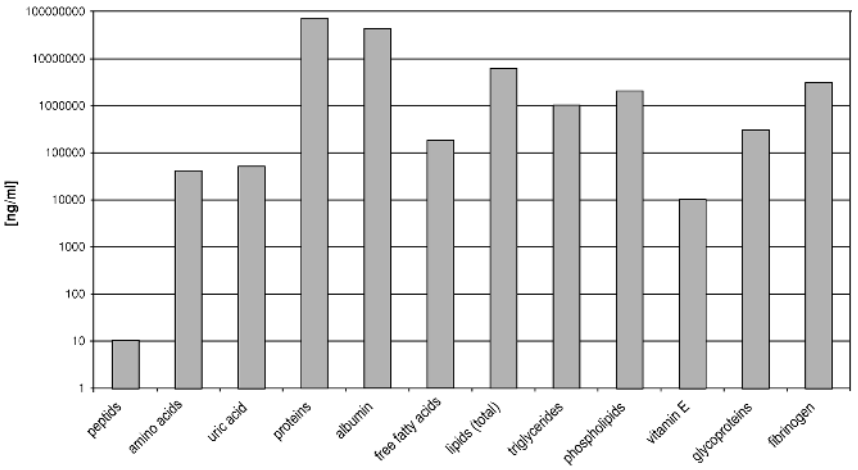
The approach described in this book develops analytical procedures starting with detection – in this case UV detection. As a rule, of thumb you can say that with a value of $E^{1\%}_{1cm}$ >200 (ε approx. 5000 according to molecular weight) you can still detect approximately 1 ng of substance as a peak in the chromatogram. This also applies to Ibuprofen at a wavelength of below 230 nm (the value referred to in the table is at approximately 260 nm and not at the maximum absorption of <240 nm – not shown in the UV spectrum).

A hint: Endogenous substances in plasma contain various functional groups and conjugated double bonds. Therefore, practice has shown: the shorter the wavelength in the UV, the more substances in plasma are to be expected, which may chromatographically interfere.

Back to Ibuprofen: If our question is to determine about 10 µg/ml plasma and about 1 ng as a peak can be seen in the chromatogram at 220–230 nm, then we would only have to inject 0.1 µL plasma unto the column. Is it so easy?

Let us now turn to HPLC separation. The obvious approach is the RP separation (reversed phase, C 8 or C 18). In order to elute Ibuprofen you need methanol or acetonitrile (ACN) in higher concentration in the mobile phase. Thus, you are not in a position to inject plasma directly because the plasma proteins due to the higher share of organic solvent in the mobile phase would be precipitated at once in the column. Therefore, not only plasma dilution (for instance 0.1 µL plasma with water/1:20) but also sample preparation are necessary. The substances contained in plasma constitute a further problem: At 220–230 nm a larger part of them absorb comparatively well. Plasma itself contains approx. 8 % (= 80 000 ppm) plasma proteins – the larger share of it as Albumin. This shows clearly that at first many unwanted substances have to be eliminated before you can succeed in determination. A successful HPLC separation plays only a subordinate part in this case in the whole determination. Ibuprofen in its undissociated form tends to be rather lipophilic. This fact leads to the recommendation of an LLE (liquid-liquid extraction) with an organic solvent not mixable with water: Medium polarity like diisopropylether, chloroform or mixtures of hexane/heptane with middle polar solvents (see Section 3.3.1.2 for more information). For example, ethyl acetate would be easy to handle, however, it absorbs several per cent of watery phase, and therefore it can usually only be used in a mixture of aliphates in order to reduce the absorption of water significantly.

A rather effective precleaning can thus be reached as follows: After addition of, e. g., H_3PO_4 to the plasma and mixing thoroughly with an organic solvent (not mixable with water), you centrifuge the sample vial and take the (usually) upper organic phase subsequently, which then contains the majority of the Ibuprofen present in the sample.



The concentration of selective substances in the plasma (attention: concentration to intervals of 10)

With this extraction not only can all hydrophilic substances be eliminated (thus also nearly all proteins, peptides and amino acids), but also all lipophilic basic substances because those are salts under these pH conditions and remain in the water phase. Thus, with the Ibuprofen all lipophilic acids and all lipophilic neutral substances are extracted. When this precleaning is not sufficient – the chromatogram shows too many disturbing substances at the elution time of Ibuprofen – then you can carry out a second extraction step: a re-extraction. In doing so the organic solvent is not evaporated but mixed with a watery basic buffer, shaken and centrifuged. Now all acids– including Ibuprofen – go into the watery phase as salts and can be – under certain circumstances – directly injected into the HPLC system. With this 2nd step of extraction all neutral lipophilic substances can be removed, they remain in the organic solvent. After such a precleaning you can very easily and even selectively gain a determination of Ibuprofen with HPLC-UV at 220–230 nm down to 10 µg/ml plasma. With fluorescence detectors, which have still enough light intensity at 220 nm, you can even get a far more selective detection for Ibuprofen[2]. An alternative sample preparation would be a solid-phase extraction (SPE): plasma slightly made acidic, is applied (preferably offline) to C 8- or C 18-material (solid-phase extraction cartridges) to which Ibuprofen adheres well. Next step: the column is eluted with methanol or acetonitrile after a washing step with water. Though the elute not only contains Ibuprofen, but of course also all lipophilic acids, all lipophilic neutral substances and also partly lipophilic bases.

A further important aspect with respect to sample preparation and clean up is the protein binding, which you must always pay attention to: most of the drugs and metabolites are subject to protein binding, a more or less strong one, mostly in the

2 Eur. J. Clin. Pharmacol. 48 (1995), 505–511 "Comparison of the bioavailability of dexibuprofen administered alone or as part of racemic ibuprofen"

manner of a Nernstsche distribution (a lipophilic gradient so to speak). As a rule, this protein binding can be dissolved easily and very quickly with liquid-liquid or with liquid-solid extraction (occasionally not so well). Ibuprofen's protein binding comprises more than 99 %. However, this is not important with the above-suggested sample preparation because the protein binding dissolves easily (see also Useful tip 6: protein binding).

Remark to determination in plasma and protein binding: An analyst can only give a determination of the total quantity of substance within plasma/serum in nearly all cases. Because the protein binding is a factor that varies and already slight modifications of plasma (dilution, change of pH, supplement of solvents) can change this factor dramatically, we must search for conditions that guarantee a safe determination of free substance together with protein-bound substance.

For instance, if you choose to eliminate proteins by precipitation with $HClO_4$ or TCA, then a big quantity of Ibuprofen (due to protein binding or being enclosed) would be precipitated, too. Thus, this determination would lead you to totally wrong results (far below the actual value). If you use protein precipitation with acetonitrile or methanol on the other hand, the protein binding is dissolved (as a rule of thumb), before the proteins are precipitated. Thus, such a protein precipitation, as far as its accuracy of recovery is concerned, is in most cases the right and safe choice. Concerning HPLC separation there is not much you must consider; of course the mobile phase has to be acidic in order to chromatograph Ibuprofen as an undissociated molecule. As a rule, you should achieve a *k'* value of 2–5. Please avoid the mistake that you think of retention time instead of *k'* values: each type of column has different dead times depending on the length of the column, column diameter and flow of mobile phase. The next decision to make is, if chromatography should be carried out isocratically or by using a gradient. This decision depends on numerous circumstances and will be discussed at length later (e. g., Section 4.1.1).

Useful tip 1: Plasma or serum? When we have a free choice, then we take either serum, which we let stand a period long enough before the centrifugation (the reason for this is to ensure that coagulation is largely completed) or plasma, that was centrifuged thoroughly. Otherwise, there will always be a risk of "fibrin clots" as we used to call them or "fibrin threads". These tend to clog up pipettes when pipetting and you lose sample volume when you transfer into other containers. Of course, you can do a second centrifugation of serum or plasma samples, but this means a further step in the procedure and is only useful if you can centrifuge in a rather strong way. The fact that you ought to wait for some time before you centrifuge full-blood (samples), may make you decide not to use human serum: On the one hand, it may distract the daily routine in hospital, on the other hand there are some substances whose successful determination demands that they are centrifuged quickly (some of them at 4 °C) and are frozen quickly in order to avoid decomposition. A general rule, which must be obeyed, is: the analyst is obliged to establish and decide after pretests how plasma/serum must be gained (time, anticoagulant, stabilizers to be added) and at which temperature it should be stored.

1.2
Second Question: Determination of Tryptophan in Urine

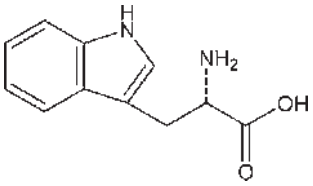
Certain therapies or conditions of illnesses change the secretion of certain hydrophilic acids into urine. Suppose, your task is to work on a method for detection of Tryptophan in urine.

According to our effective way of doing things we start again with questions as follows

- Which limit of determination is desired?
- Which kind of device is at your disposal?

For our given example we suppose that a limit of determination of 1 µg/ml urine is required and that, although an HPLC-MS-device (single quadrupole) is available in your laboratory, it is, however, needed for other research most of the time and thus cannot be used. Should this subject (determination of Tryptophan in urine) turn out to be effective, it might lead you to think of using that device, too. But for the time being you have got to do things merely with an HPLC-UV (variable wavelength/DAD) as well as an old electrochemical detector.

As a next step we consider molecular structure, solubility, UV spectrum and electrochemical behavior as well as MS-ionization (for future steps) of Tryptophan respectively.



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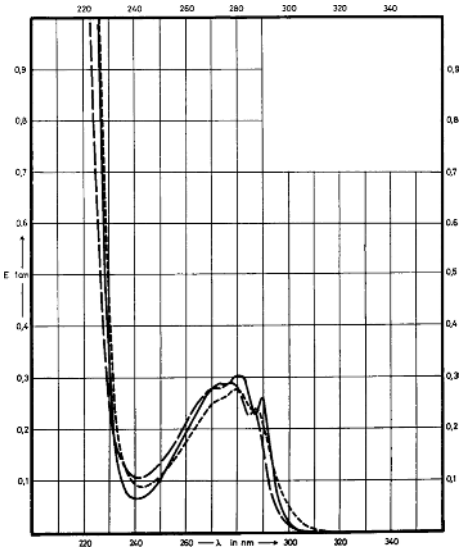


Table 1.2: Tryptophan – Details to the UV-Spectrum

solvent **symbol**	**methanol** ———	**water** – · – · –	**0.1M HCl** – – – – –	**0.1M NaOH** · · · · · ·
absorption maximum	290 nm 280 nm		286 nm 278 nm	288 nm 280 nm
$E^{1\%}_{1cm}$	259 303		234 290	238 275
ε	5290 6190		4780 5920	4860 5620

- Structure: Tryptophan is definitely an amphoteric molecule – as are all amino acids – thus it is hardly extractable. It has got some lipophilic structure parts – so it is extractable with SPE (C 8 or C 18). It contains two essential functional groups: -NH 2 and -COOH (-NH in the ring tends to be subordinate).
- Solubility: generally speaking it dissolves well in water, alcohols and acetonitrile.
- UV spectrum: absorbing well at 280 nm (and thus it does not present a problem using gradient elution with acetonitrile or methanol).
- Electrochemistry: the possibility of oxidation of the -NH in the ring (technical literature contains reliable details on oxidation of Tryptophan).
- MS: probably good ionization yield under positive conditions (if necessary ask a colleague who has more experience within MS).

I cannot argue for mass-spectrometric detection – besides the fact that you have not got an instrument at your disposal – because ionization and detection with mass spectrometry yield as a rule less reproducibility and accuracy compared to UV detection unless using a chemically extremely similar internal standard to the substance (stable isotope labelled substance preferably). However, such an internal standard can be rather expensive and in cases of novel substances there might not be such a substance commercially available (for further information look up Section 2.3.6). Arguments against electrochemical detection: only a few analysts have still got experience with such a detector today. The handling of the instrument is distinctly more complicated than that of a UV detector, however, its selectivity and sensitivity are sometimes excellent. Besides, compared to MS its costs are very reasonable.

So we will try to solve the question with HPLC-UV. At 280 nm Tryptophan shows an $E^{1\%}_{1cm}$ of 303, i. e. 1 ng of substance can be easily detected as a peak and this at a distinctly more selective wavelength than for Ibuprofen determination in the last chapter. So we would have to apply 1 µL of urine or the substance out of 1 µL urine onto the HPLC column. Which column is recommended? As Tryptophan has a

certain lipophilic structure, you can use C 8 or C 18 columns with 5–15 % methanol or acetonitrile. For safety reasons we recommend you to take an AQ-column (various manufacturers offer reversed-phase materials, which you can run in a watery phase for a longer period of time without the separation process collapsing being caused by lack of organic solvent). You could also use an ion-exchange column, whereby cationic exchangers (binding the -NH_2 of Tryptophan) are to be preferred to anionic exchangers (binding the -COOH of Tryptophan), as we have found out. However, because the use of and the experience with ion exchangers are rare in analytical laboratories, I rather recommend to you use C 8 or C 18 columns.

Sample preparation is hardly feasible (only strong RP materials like ENV+ (an offline SPE cartridge) or ion exchangers are suitable for it), thus it is necessary to inject urine directly. As a general rule with urine (especially deep-frozen urine), you have to be careful that all compounds are solved again after thawing (often it only helps to warm it to 37 °C and to shake it or to use ultrasound).

You must always consider to take a representative sample (which is distinctly easier if you have got plasma!). In order to avoid substances precipitating again you need to dilute 50 µL urine with, e. g., 250 µL water (perhaps you take 5 % of methanol in water, so that also more lipophilic substances remain dissolved). Then you could inject 10–20 µL unto the HPLC column.

As practice shows, two problems can arise:

1. If you succeed in gaining a selective method isocratically (that means a narrow peak appears). This peak only gains height if you add similar quantities of Tryptophan as a pure substance to your urine sample. But presumably in the next or at the latest in the 5th chromatogram of urine injection a problem will show up: broad signals of lately eluting peaks (from former injections) will disturb you in your determination. What you can do to counter this, is to apply a "gradient system" and after the isocratic separation to switch to an organic share of 80–90 % for 1–3 minutes, so that disturbing substances get rinsed out quickly from the column.
2. If separation is unselective (the peak of a pure Tryptophan solution in the chromatogram is narrower than in urine after adding a pure Tryptophan solution to the urine sample), you ought to repeat separation with RP columns of various manufacturers, in order to get a better selectivity.

An alternative and often better strategy for molecules like Tryptophan is to carry out an ion-pairing chromatography. I assume that you have already had to work with buffer systems when you used C 8/C 18 materials or TFA in the mobile phase, to obtain a good peak shape. In the given example you can use, e. g., pentane sulfonic acid (approx. 10 mM) in 10–20 % acetonitrile or methanol in the mobile phase (you need certain parts of organic solvents for solving pentane sulfonic acid and for the elution).

Pentane sulfonic acid and Tryptophan together form an ion pair under acidic conditions (using the -NH_2 of Tryptophan). This ion pair requires a distinctly higher percentage of organic solvent for elution than Tryptophan alone.

Are the disturbing substances only composed of organic acids derived from urine (which is probably the case), then these do elute much sooner as the ion pair and will not disturb the determination of Tryptophan any longer. However, I assume that an analogous step of rinsing out disturbing substances, which elute later and would disturb the determination of Tryptophan in a later chromatogram, by a gradient is necessary.

1.3 Third Question: Determination of Paclitaxel in Tissue

It is possible that you are requested to determine certain substances in different tissues. We presume that the analyst has gained sufficient experience with plasma and urine as matrix, but none with tissue. The decisive factors for determination in tissue are:

- homogenizing of the tissue;
- reaching a high recovery rate of the substance to be detected.

Useful tip 2: Tissue sample preparation As we have already analyzed very many different types of tissues successfully, I would like to mention a few general rules here (for more details see Section 11.19).

You have to distinguish between tissue that is soft, nearly without fibers and other kinds of tissues. Soft tissues such as brain or often also liver, can be very well homogenized with an Ultraturrax. In doing so the procedure is as follows: After you have cut the defrosted tissue (it is not always easy to take a representative sample), a part of it is weighed into, e. g., a plastic centrifuge vial and mixed with 2–3 parts of ethanol, watery ethanol or acetone (depending on the substance to be determined and the matrix) and then homogenized with the Ultraturrax within 30–60 s (longer times can warm up the sample to higher temperatures). A homogeneous share of this blended tissue or this suspension is then weighed into, e. g., a small vial and diluted several times with water or certain hydrophilic solvents, so that you obtain approximately a 10 % share of protein – which is similar to plasma then. Then, use a procedure following the principles used with plasma.

For all other kinds of tissue I recommend to use, e. g., a so-called Dismembrator, which works in principle in this way: thus with an iron ball or a similar tool you pulverize the – due to liquid nitrogen – frozen tissue within about 30 s. There is rarely a tissue that cannot be pulverized with that procedure at such low temperatures.

The recovery of a given substance from tissue is always tested in two steps except for endogenous substances:

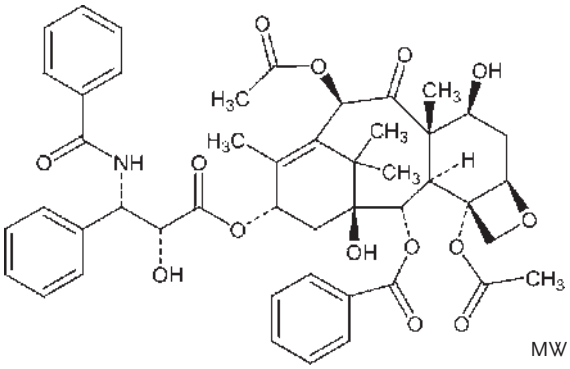
1. First, you add to a part of the homogenized blank tissue the substance in question in sufficient concentration (the concentration must not be near the determination limit, because otherwise the results would vary too much and would not be representative). Then apply your sample preparation. Do that also with a solution without tissue and compare the results. Only when you got a high recovery rate (to obtain that sometimes requires many and various pretests), can you take the next step.

2. If there already exists a tissue from preclinical or clinical application, you can vary the extraction conditions, which you have determined before with several similar homogenates to obtain a maximum yield.

A marginal note: in case these extractions ought to be analyzed with HPLC-MS or MS/MS you have to be aware that immense matrix effects can occur, which may suppress ionization of certain substances up to a remarkable 95 %. A brief discussion concerning an important matter: Irrespective of transferability of sample preparation from plasma to tissue the recovery from tissue may appear to be completely different (that means it may be worse). Occasionally, things turn out to be alright, when we used various detergents, if the recovery was a difficult matter.

There are nonionic and ionic tensides, relatively hydrophilic as much as hydrophobic ones. To apply tensides can bring forth a miracle, but pay attention to the fact that you get rid of the larger share of tensides, before you carry through the HPLC analytics. Large quantities of tensides can not only influence the HPLC separation, but also suppress the MS signal so much that you will not be able to recognize your substance of interest. In such cases especially it is crucial to determine, if recovery is still low or if strong matrix effects occur.

To sum up: Clinical analytics aims at obtaining safe and accurate results by applying methods that can be developed by good analysts in a relatively short time. This book shall be one tool to foster that ability.



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Paclitaxel is a molecule with a medium polarity (for further details: requested determination limit, structure and solubility, see Section 11.14 or the Appendix). The tissue is cut into small pieces (take representative sample!), deep frozen for 15 min in liquid nitrogen with an iron ball contained in a small Teflon box and then smashed with a Dismembrator for about 30 s. A small portion of the sample (5 mg out of 30 mg, approximately) is mixed with 100 µL of 50 % ethanol, 20 µL of Triton X 100 (5 % in water) and 300 µL water; and then you can extract (LLE) with 4 ml diisopropylether. If you do not add ethanol you will have difficulty concerning the solubility of the substance to be analyzed and – without Triton X 100 (detergent) – the recovery with certain tissues will turn out poorly.