At its 152nd session (June 2015), the European Pharmacopoeia Commission approved the publication of a new edition of the Technical guide for the elaboration of monographs (7th Edition) which is available on the EDQM website. In this context, improvements have been made in the section on response and correction factors where a detailed explanation is now provided on how these factors should be determined.

The corresponding section in the 7th Edition of the Technical guide is an extract of the following article which provides more detailed information on the definition and determination of response and correction factors and shows examples of challenges the analyst may be confronted with and how these can be overcome.

Response and correction factors in monographs of the European Pharmacopoeia

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Introduction

Most monographs in the European Pharmacopoeia describe a test for related substances by liquid chromatography using UV detection [1]. These tests serve for the determination of impurities which may be educts or by-products of the synthesis or degradation products.

The more recent monographs distinguish between specified and unspecified impurities [2], where an individual acceptance criterion exists for specified impurities and their unequivocal identification in the chromatographic system is therefore necessary. This identification shall not be confused with the identification in the sense of elucidation of the structure, it may therefore be better to use the expression ‘peak identification’. The latter is preferably carried out using chemical reference substances (CRS) which may be individual impurities or, due to the unavailability of sufficient amounts of the individual impurities, mixtures of the monograph substance (= substance to be examined, hereinafter called test substance) with the specified impurities or mixtures of the impurities without addition of the test substance [3].

The monograph then not only provides the means to identify these impurities in the chromatographic system, but also describes how they are quantified.

Quantification

The amount of impurities present in a sample is usually determined using the method of ‘external standardisation’, but peak area normalisation is also employed.

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When the method of external standardisation is described, then the preferred option is to use the individual impurities as reference substances by preparing reference solutions containing these impurities at concentrations which should preferably be in the range of the specification of the impurity, i.e. often between 0.1 and 1.0 %, in relation to the concentration of the test solution.

However, as mentioned above, these impurities are often not available in sufficient amounts to be established and used as individual reference standards. In such a case a dilution of the test solution, e.g. 1:100, may be used as an external standard to determine the amount of these impurities in the test sample. This option can be chosen when the test substance shows an absorption curve similar to that of the impurities, i.e. the detector response is similar at the chosen wavelength. On the other hand, different absorption curves may lead to markedly different responses at the chosen wavelength. It is therefore necessary to determine the response factors of the specified impurities during the validation of the method. Also, the optimisation of the choice of a suitable detection wavelength can be useful.

Definition of response and correction factors

As already discussed in the literature [4], the meaning of the expressions ‘response factor’ and ‘correction factor’ is often handled in a different way. According to the European Pharmacopoeia [5], the response factor (or relative response factor, *RRF*) expresses the sensitivity of a detector for a given substance relative to a standard substance, i.e. the ratio of the detector response of the impurity to the detector response of the test substance at the same concentration. The correction factor given in the monograph is then the reciprocal value of the response factor and the area of the impurity peak in the chromatogram obtained with the test solution must be multiplied by this correction factor. Generally, the response factor can be calculated using the following expression:

$$RRF = (A_i/A_s) \times (C_s/C_i)$$

RRF = response factor;

A_i = area of the peak due to the impurity;

A_s = area of the peak due to the test substance;

C_s = concentration of the test substance in mg/mL;

C_i = concentration of the impurity in mg/mL.

For the calculation, the mean of the area ratios over the whole range of linearity or a comparison of the slopes of the linearity regression equation, impurity versus test substance, may be used.

The meaning and the correct use of response and correction factors are also discussed in the Technical guide of the European Pharmacopoeia [6]. According to general chapter *2.2.46. Chromatographic separation techniques* and the Technical guide, correction of the area of an impurity peak becomes necessary when the response of the impurity is outside the range of 0.8 to 1.2 compared to the test substance, i.e. differences in response are not taken into account within the range of 0.8 to 1.2. As a consequence, in all cases where, for a specified impurity, a response factor outside this range has been detected during the validation of a monograph, i.e. where the correction factor is also

outside this range, a corresponding correction factor is stated in the related substances section of the monograph.

However, no guidance is given on how to determine response and correction factors.

Determination of response factors

The response factor can be determined by preparing solutions of defined concentrations of impurity and the test substance, by chromatographing these solutions at a given wavelength and flow rate and subsequent comparison of peak areas using the formula depicted above. Ideally, the concentration of the impurity and that of the test substance should be in the same order of magnitude and the measurement should be carried out using a calibration curve determined at several points around the concentration which corresponds to the acceptance criterion of the impurity.

However, it appears of major importance that not only the corresponding amounts of impurity and test substance are weighed and peak areas compared, but that the weighings are corrected for the purity of the substances examined. Ideally, the chromatographic purity, water content and content of residual solvents of the impurity and the test substance should be tested. A provisional value will be assigned on the basis of the following formula, which would follow the same procedure as used for the value assignment to a reference standard [3]:

content (%) = [100 – (water + solvents)] x chromatographic purity in per cent/100

The terms C_s and C_i in the above formula are then corrected for the content determined for the impurity and the test substance:

$$RRF = (A_i/A_s) \times (C_s \times P_s/C_i \times P_i)$$

RRF = response factor;

A_i = area of the peak due to the impurity;

A_s = area of the peak due to the test substance;

C_s = concentration of the test substance in mg/mL;

P_s = provisionally assigned content of the test substance in per cent/100;

C_i = concentration of the impurity in mg/mL;

P_i = provisionally assigned content of the impurity in per cent/100.

As the determination of the response factor represents a part of the validation of the method it should be subject to verification in at least 2 laboratories, preferably using the same protocol so that comparable results can be obtained.

A limiting factor in the testing is often the amount of impurity available. Whilst it is desirable to determine the content/purity of the impurity in the same manner as an impurity reference substance used as external standard is established, the amount available is often scarce. It is then at the discretion of the analyst to choose such methods which consume small amounts of substance, such as thermogravimetry or coulometry for the determination of water and/or solvents, so that scientifically valid results can still be obtained. It may also be useful to determine the response factor using different types of detectors.

A further important issue to be considered by the analyst is the form (base/acid or salt) of the impurity and the test substance which is used when the response factor is determined. As long as both exist in the same form, i.e. base/base or salt/salt, the weighed quantities of the samples can be used as such but where there are differences, an additional correction factor for the molecular mass ratio should be introduced in the calculation.

For example, if the monograph substance is used as a base and the impurity as a tartrate salt (i.e. with a counter-ion of considerable molecular mass), then the amount of the impurity weighed should be multiplied by the correction factor M_{salt}/M_{base} and corrected for the difference in molecular mass.

Restrictions and particular aspects in the determination and use of response factors

Different absorption maxima

Ideally the absorption maxima of the impurity and the test substance should be at wavelengths which are not too different, so that the measurement of the responses at a given wavelength takes place at or close to the absorption maximum of each substance. When the absorption curves of the impurity and test substance are very different, then the response of the test substance may be measured at its maximum but for the impurity it may be measured on the slope of the absorption curve. As shown in Figure 1, measuring at the maximum of one of the absorption curves would mean simultaneously measuring on the steep part of the second curve. As a consequence, small variations in the accuracy of the wavelength measurement may have a strong impact on the absorbance determined for the impurity whereas the absorbance measured for the test substance is almost unchanged.

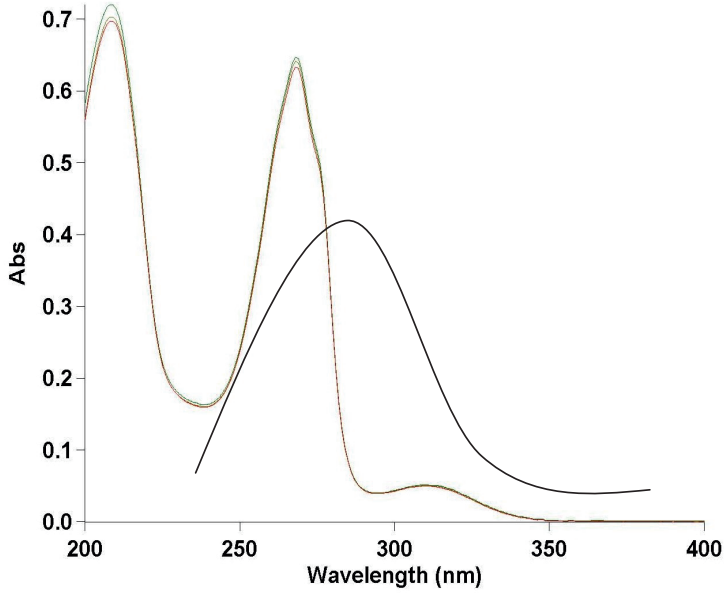

Figure 1 – *Absorption curves of impurity and test substance with maxima at different wavelengths*

In this context it should be mentioned that general chapter *2.2.25. Ultraviolet and visible absorption spectrophotometry* [7] describes a permitted tolerance for the verification

of the wavelength scale whereas chapter *2.2.29. Liquid chromatography* includes a paragraph on detectors where, however, no control of wavelength scale or absorbance is described.

An elegant solution to overcome this problem has been found for the monograph on *St. John's Wort dry extract, quantified* [8]. In this monograph, the content of hyperforin is determined by LC using *rutoside trihydrate CRS* (= rutin) as assay standard with an assigned content. To this purpose it was necessary to describe a correction factor for hyperforin relative to rutin. At the absorption maximum of hyperforin at 275 nm, the UV-curve of rutin shows a steep absorption flank, i.e. the situation is as described above. For this reason the correction factor has been determined by measuring the responses at 2 different wavelengths, at 360 nm for rutin and 275 nm for hyperforin, and the wavelengths are switched from 360 nm to 275 nm during the chromatographic run (at 22 min) so that rutin and some flavonoids are detected at 360 nm and the late eluting hyperforin and adhyperforin at 275 nm (Figure 2).

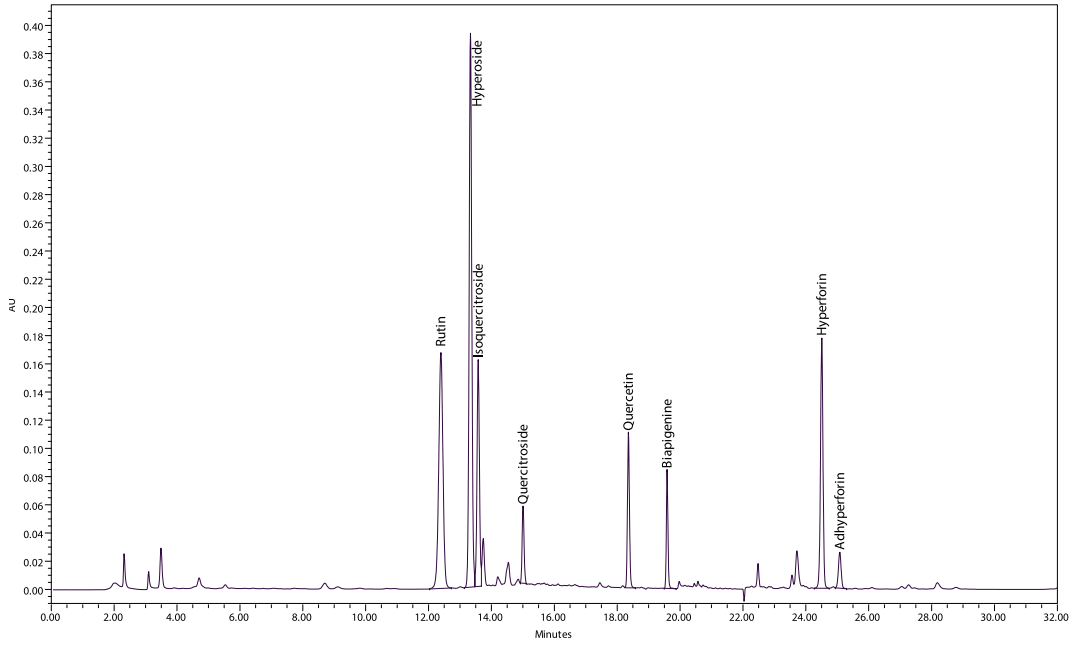

Figure 2 – *Typical chromatogram obtained with St. John's wort dry extract HRS in the assay for hyperforin and flavonoids*

Low response of impurities

A particular problem is observed when the response of the impurity is much lower than that of the test substance, i.e. the area of the impurity peak must be multiplied by a correction factor significantly greater than 1. In this case it may occur that a specified impurity is present at a level above the reporting threshold (disregard limit, usually 0.05 %) but, due to the low response, the corresponding peak appears clearly below that limit and may therefore be disregarded when the reporting threshold is applied before the application of the correction factor.

For example, an impurity with a correction factor of 10 might be present at a level of 0.2 % but erroneously disregarded because the corresponding peak only appears at a

0.02 % level before multiplying by the correction factor, i.e. below the usual reporting threshold of 0.05 %.

The peak area must therefore be multiplied by the correction factor before applying the reporting threshold. A sensitivity test may be described in the monograph. To this purpose it can be envisaged to describe a reference solution containing the impurity at an amount corresponding to the impurity disregard level and require a minimum signal-to-noise ratio (*S*/*N*) of 10, corresponding to the limit of quantification for the peak obtained. If the quantification is done using a diluted test solution as external standard, then the *S*/*N* of a peak obtained with a diluted test solution at a concentration corresponding to the reporting threshold should be at least 10 x the correction factor (e.g. correction factor is 4, then the *S*/*N* requirement should be at least 40). In cases where the sensitivity is sufficiently good and the impurity peak was shown to have an *S*/*N* value far greater than 10 at the reporting threshold, this test may not be necessary.

Limitations in the use of response/correction factors

As described above, low responding impurities requiring a high correction factor may represent a limitation in that the limit of quantification may be higher than the reporting threshold for the individual impurity. In that case, a different detection wavelength should be chosen. In general, the accuracy of the quantitative determination decreases when a high correction factor is used because the peak area repeatability for several injections of a reference or test solution is not as good for small peaks as for big peaks. The uncertainty of the determination therefore increases the higher the correction factor is. For impurities with a high response factor, i.e. correction factor below 1, this problem does not arise.

For this reason the Technical guide of the European Pharmacopoeia [6] recommends a lower limit of 0.2 for the response factor. Below this value use of the impurity as external standard or measurement at a different detection wavelength is to be considered.

Conclusion

The use of response and correction factors for the determination of impurities by liquid chromatography represents a valuable method when the impurity concerned is not available in sufficient amounts to be used as external standard and the response factor is outside the range of 0.8 to 1.2. Particular care must be taken for the correct determination of the response factor in order to obtain reliable results for the determination of the amount of impurity present in a sample. Restrictions for this approach are very low response factors and absorption maxima very different from the maximum of the substance to be examined. This document gives examples on how these problems can be solved.

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