Journal of Chromatography, 289 (1984) 333–337
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 313

PROBLEM OF THE INTERNAL STANDARD IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

M. VERZELE*, L. USE and M. VAN KERREBROECK
Laboratory of Organic Chemistry, State University of Ghent, Krijgslaan 281 (S. 4), B-9000 Ghent (Belgium)

SUMMARY

A general approach for finding a suitable internal standard in high-performance liquid chromatography is presented. A mixture of the anilides of hydrolysed perhydrogenated fat (fatty acids from C_8 to C_{18}) is chromatographed under the conditions of the analysis. Deciding which particular anilide to choose is then generally easy. Optimum wavelengths for UV detection can be selected by changing the substituents on the anilide aromatic nucleus. The anilides are stable in dilute solution, unlike many other compounds suggested as internal standards.

INTRODUCTION

It is generally difficult to find a suitable internal standard in high-performance liquid chromatography (HPLC), as illustrated by a recent discussion by Guillemin *et al.*[1]. They proposed the deferred standard technique, in which the compound to be analysed (or another suitable compound) is injected in pure form, some time after but during the chromatographic run with the real sample.

This method of Guillemin *et al.* is a variation of the external standard method and consequently suffers from the drawbacks of this approach. The internal standard technique has also been discussed critically by Haefelfinger[2]. His conclusion was that the external standard technique can just as well be used, considering the high precision of HPLC sample loop injection.

We do not agree with this. If at all possible, we believe that the internal standard technique is the method of choice. The interest in alternatives and the recent criticisms in our opinion reflect the difficulty of finding a suitable internal standard.

EXPERIMENTAL

The synthesis of anilides is straightforward. The acid chlorides are prepared by adding the appropriate acid to a 1.5 molar excess of thionyl chloride and are isolated by fractional distillation. For higher acid chlorides vacuum distillation is necessary. The acid chlorides are diluted by adding about five times the weight of methylene chloride and to this solution is added an equimolar mixture of the aniline

0021-9673/84/$03.00

and triethylamine. The methylene chloride solution is washed repeatedly with dilute acid to remove underivatized aniline and triethylamine and the final solution is washed with water until acid-free. Final crystallization of the anilides is carried out in mixtures of isooctane and methylene chloride. Two pure anilides were prepared: the *p*-nitroanilide of myristic acid (m.p. 89°C; $E_{1\%}^{1cm}$ at 314 nm = 420) and the *m*-nitroanilide of palmitic acid (m.p. 95–96°C; $E_{1\%}^{1cm}$ at 336 nm = 35.2).

To obtain a mixture of fatty acids, perhydrogenated fat was hydrolysed with 4 *N* methanolic potassium hydroxide (80:20). The mixed fatty acids were converted into the anilides as described above. Such a mixture of anilides changes in relative composition on each crystallization. To retain some C_8 and C_{10} anilide, the mixture should be crystallized only once. A chromatogram of such a mixture of *p*-nitroanilides is shown in Fig. 1.

Full chromatographic details are given in the captions of the figures.

RESULTS

The quantitative analyses of greatest interest in this laboratory are those of bitter acids in hops and in beers. As a good measuring wavelength for α-acids is at 314 nm, a *p*-nitroanilide is indicated as an internal standard. Co-chromatography as

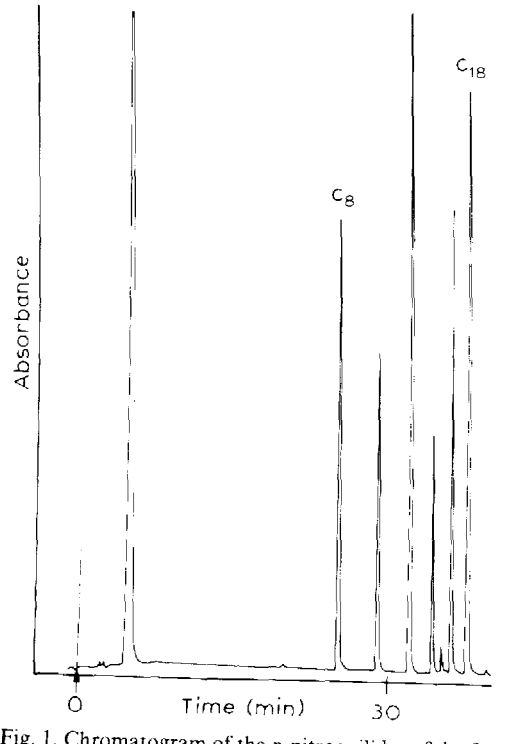

Fig. 1. Chromatogram of the *p*-nitroanilides of the fatty acids of hydrolysed hydrogenated fat. Varian LC 5020 chromatograph. Column, 25 × 0.46 cm I.D., packed with 5-μm ROSiL-C_{18}-HL-D (an octadecylated spherical silica gel from RSL-Alltech). Gradient elution with methanol water, 50:50 to 100:0 in 30 min at 1 ml/min. Detection at 314 nm. The first peak is *p*-nitroaniline.

discussed above showed that the *p*-nitroanilide of myristic acid was the most suitable[3].

Another analysis involves the oxidation products of β-acids in beer[4]. For this the best measuring wavelength is 280 nm and an anilide can therefore be chosen as the internal standard. The anilide derived from lauric acid has a suitable retention time, established by running the sample to be analysed and the hydrogenated fat anilide mixture under identical conditions. Another analysis developed in our laboratory is the determination of residual α- and β-acids in beer[5]. The *m*-nitroanilide of palmitic acid was found to be the most suitable internal standard with a measuring wavelength of 336 nm.

The above examples are for reversed-phase HPLC systems. For more polar stationary phases, *e.g.*, normal-phase silica gel, the anilides can also be used as internal standards. Their retention time, however, is much shorter and a more strongly retained compound may be desirable. For this the anilides derived from benzoic acid are suitable. An example of the possibility of the normal-phase determination of piperine is shown in Fig. 2. For details of pepper and piperine determination by HPLC we refer to earlier papers from this laboratory[6,7].

DISCUSSION

Chemicals that are stable in bulk can be reactive in dilute solution, and this

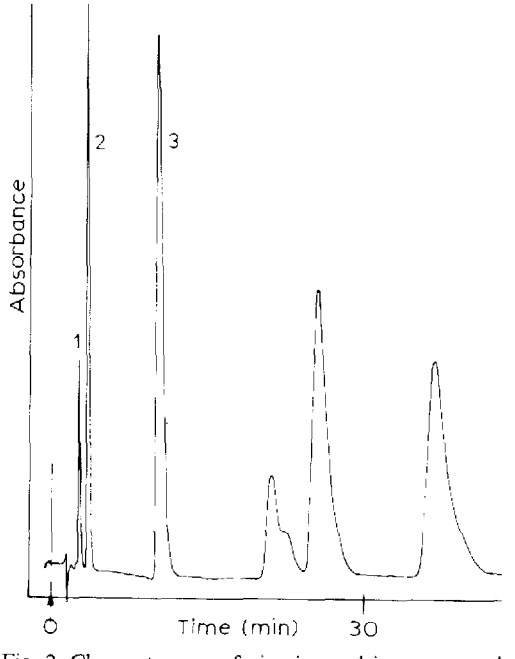

Fig. 2. Chromatogram of piperine and isomers together with some anilides. 1 = *p*-Nitroanilide of C_{14} acid; 2 = anilide of C_{12} acid; 3 = *p*-acetylanilide of benzoic acid. Other peaks: chavicine, isochavicine, isopiperine and (last) piperine. This mixture is the photostationary isomerization mixture of piperine. Varian LC 5020 chromatograph. Column, 25 × 0.46 cm I.D., packed with 10-μm RSiL (a non-spherical silica gel from RSL-Alltech). The silica gel was treated with a citric acid–phosphate buffer as described[6,7]. elution with dry methylene chloride 0.1% 2-propanol at 4 ml/min. Detection at 280 nm.

applies to the internal standards in HPLC. An example is the chalkone standard used in our method for the analysis of hops acids[8]. Photoisomerization of the chalkone *trans* form into a *cis–trans* mixture occurs in dilute solution, even in diffuse laboratory light. Using aluminium foil to shield the light prevents this. A similar reaction was reported by Lance *et al.*[9] for the 2,4-dinitrophenylhydrazone of octanal or decanal. Oxidation must also be suspected as a reason for instability of internal standards. In dilute solution and in a transparent solvent (usual in HPLC for UV detection compatibility), oxidation can be photocatalysed. An unexpected case occurred when we tried to establish the stability of lauric acid anilide by monitoring its HPLC peak intensity against that of anthracene. If the anilide were stable, the ratio of the peak intensities should remain constant with time, and on varying one of the concentrations a graph passing through the origin should be obtained. We found that it is the anthracene which disappears, very rapidly, and not the anilide. Anilides were therefore investigated as possible internal standards.

Anilides combine the stability of the amide bond and the UV activity of the aromatic ring. The amide bond is a polar function and therefore anilides are polar compounds with sufficient apolar parts to ensure equally acceptable solubility in polar and apolar solvents, which can be important. For the analysis of the bitter acids of beer by direct injection of beer, the chromatographically acceptable standard 2,6-di-*tert.*-butylphenol, for example, could not be used internally because of solubility problems in aqueous media[10]. The retention time and therefore the position of the anilide internal standards in the chromatogram can be varied by changing the length of the chain of the carboxylic acid moiety of the anilide The most easily accessible straight-chain acids are the even-carbon-numbered natural fatty acids. If a smaller shift in retention time than is available between two consecutive homologous acid anilides is desired, another anilide can be chosen. All nitroanilides, for example, elute later than the corresponding anilides on a reversed-phase column, the *ortho* derivatives having the longest retention times. Many problems can be solved with the six even-carbon-numbered straight-chain carboxylic acids between C_8 and C_{18}. Commercial perhydrogenated fat yields virtually only these acids on hydrolysis. The idea is therefore to (co)chromatograph the anilides of that mixture in order to establish which particular anilide is the most suitable as an internal standard.

A further problem with internal standards for HPLC in the usual UV detection mode is the choice of the detection wavelength. It is rarely possible to choose or find a wavelength at which both the analyte and the internal standard have a maximum,

TABLE I

WAVELENGTH OF MAXIMUM ABSORPTION AND OPTIMUM WAVELENGTH RANGE OF SOME SELECTED ANILIDES

Compounds	λ_{max} *(nm)*
Anilides of fatty acids	244 (240–290)
o-Methoxyanilides of fatty acids	280 (265–290)
p-Acetylanilides of fatty acids	304 (280–310)
p-Nitroanilides of fatty acids	314 (310–320)
m-Nitroanilides of fatty acids	320 (300–330)
o-Nitroanilides of fatty acids	340 (300–350)

a minimum or a plateau, where a small error in wavelength selection does not change their ratio of absorption. Wavelength selectors for HPLC instrumentation can easily be a few nanometres off-target. Simple ways of checking this have been discussed before[8]. With anilides this problem can be minimized. By varying the substituents on the aromatic ring a suitable UV range can be covered. We investigated derivatives of aniline, *o*-methoxyaniline, *p*-acetylaniline and the three nitroanilines. The UV maxima for the major long-wave absorption band of the anilides are given in Table I. Concentrations of 0.05 (*p*-nitroanilides) to 0.3 mg/ml (*m*-nitroanilides) are necessary for obtaining these long-wave absorption bands.

ACKNOWLEDGEMENTS

We thank the Ministerie voor Wetenschapsbeleid, the Nationaal Fonds voor Wetenschappelijk Onderzoek —NFWO and the Instituut voor Wetenschappelijk Onderzoek in Nijverheid en Landbouw— IWONL for financial help to the laboratory. M.V.K. thanks the IWONL for a grant for the preparation of his Dr.Sc. degree.

REFERENCES

1 C. Guillemin, J. Gressin and M. Caude, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1982) 128.
2 P. Haefelfinger, *J. Chromatogr.*, 218 (1981) 73.
3 M. Verzele, C. Dewaele and M. Van Kerrebroeck, *J. Amer. Brew. Soc.*, 41 (1983) 36.
4 M. Verzele, C. Dewaele and N. Van de Velde, *J. Amer. Brew. Soc.*, 41 (1983) 57.
5 M. Verzele, M. Van Kerrebroeck, C. Dewaele, J. Strating and L. Verhagen, *J. Chromatogr.*, (1984) in press.
6 M. Verzele and S. Qureshi, *Chromatographia*, 13 (1980) 241.
7 M. Verzele, P. Mussche and S. Qureshi, *J. Chromatogr.*, 172 (1979) 493.
8 M. Verzele, J. Van Dijck and H. Claus, *J. Inst. Brew., London*, 86 (1980) 9.
9 D. Lance, T. Kavanagh and B. Clarke, *J. Inst. Brew., London*, 87 (1983) 225.
10 M. Verzele, C. Dewaele and M. Van Kerrebroeck, *J. Chromatogr.*, 244 (1982) 321.