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Computer assisted internal standard selection for reversed-phase liquid chromatography

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ABSTRACT

A convenient method to place an internal standard in a reversed-phase liquid chromatogram has always been a challenge to the analytical chemist. Generally, this has been accomplished by trial and error or the use of a homologous series of compounds. With a database of 90 potential internal standards arranged in a retention time order, and the use of a marker solution, this selection process can be greatly simplified. The marker solution and the sample for which an internal standard is desired are injected into the liquid chromatograph and chromatographed under the same gradient conditions as those used for the database. The retention times of the marker solution for a given column are entered into the computer to rearrange the database. The sample chromatogram is examined for "open windows" and the computer is requested to suggest internal standards. This approach applies to aqueous acetonitrile eluents but is essentially column-manufacturer independent. Because of changes in selectivity with aqueous methanol or aqueous tetrahydrofuran eluents, the approach may not apply as described. However, the database should make the internal standard selection for these mobile phases much easier.

INTRODUCTION

The placement of an internal standard in a reversed-phase liquid chromatogram has always been a time-consuming operation for the analytical chemist. Methods that make use of a homologous series of compounds [1] are generally used. From a database of 90 potential internal standards, arranged in retention time order, and the use of a marker solution, this selection process can be greatly simplified. This approach applies to aqueous acetonitrile eluents but is essentially column-manufacturer independent. Because of changes in selectivity with aqueous methanol or aqueous tetrahydrofuran eluents, the approach may not apply as described. However, the database should make the internal standard selection for these mobile phases much easier. A list of criteria that an internal standard should possess is described elsewhere [2].

EXPERIMENTAL

Materials

A Hewlett-Packard Model 1090A liquid chromatograph with photodiode-array

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detector and the following reversed-phase liquid chromatography columns were used in the study: Nova-Pak C_{18}, 150 × 3.9 mm I.D. (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.); Partisil 5 ODS-3, 100 × 4.6 mm I.D. (Whatman, Clifton, NJ, U.S.A.); Spherisorb ODS-1, 3 μm, 100 × 4.6 mm I.D. (Phase Separations, Deeside, U.K.); Zorbax ODS, 150 × 4.5 mm I.D. (MAC-MOD, Chadds Ford, PA, U.S.A.). The chemicals were purchased from various chemical supply houses and were used without further purification. Stock solutions of each were prepared by dissolving 10 to 15 mg of each in 10 ml of acetonitrile. These solutions were used without further dilution to obtain retention times and spectra. Because publication of the spectra in this report would require too much space, they are not included. However, copies are available upon request from the authors.

Methods

The potential internal standards were separated by gradient elution. The "A" reservoir contained 0.005 *M* aqueous sulfuric acid and the "B" reservoir contained acetonitrile. A linear gradient from 20 to 100% B in 15 min was employed. For those compounds not eluted in this time interval, elution was continued at 100% B. A 3-min reequilibration was used. The flow-rate was 1 ml/min and the injection volume 2 μl. The chromatograms were monitored at 254 nm and a full-scale absorbance setting of 0.064 a.u. was used.

The marker solution retention times will be identical, whether water or 0.005 *M* (aq.) H_2SO_4 is used in the "A" reservoir. The use of the dilute acid is only required if acidic components are present in the sample under study.

Samples of 50 mg of acetanilide, benzophenone, α-tetralone, and 30 mg of trans-stilbene were dissolved in 50 ml of acetonitrile. A chromatogram of the marker solution is shown in Fig. 1.

The 90 potential internal standards were injected on the Nova Pak C-18 column and chromatographed as described previously. They were arranged in retention time order and entered into a terminal-format file to be ready for the BASIC program.

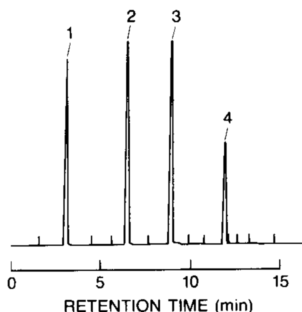


Fig. 1. Liquid chromatogram of marker solution. The marker solution containing components: 1 = acetanilide, 2 = α-tetralone, 3 = benzophenone and 4 = *trans*-stilbene as chromatographed on Nova-Pak C_{18} 150 × 3.9 mm I.D., with a linear gradient from 20 to 100% B in 15 min, where A = 0.005 *M* aq. H_2SO_4 and B = acetonitrile. The flow-rate was 1 ml/min with detection at 254 nm for an injection volume of 2 μl. Signal range was 0.064 a.u.f.s.

The BASIC program

The program, Window, was written in VAX BASIC (version 3.3) and compiled to run on VAX/VMS, version 5.0 (Digital Equipment Corp.). Each standard material required two lines in the data file: chemical name, followed by retention time. Since the BASIC program uses Linput statements when reading the data file, chemical names can include punctuations and spaces. A terminal-format file was chosen to facilitate editing of the standard data. Window only accesses the data file for input, and cannot alter reference data.

The 15-min span of standard retention times is divided into four segments with one marker compound acting as a relative retention standard for each segment. The segment boundaries (start and end times) and the associated standard for that segment are identified in Data statements in the BASIC program. The relative retention times for each standard are calculated by finding the ratio of actual marker retention time to the stored marker retention time. The retention time for each component in a segment is then multiplied by the corresponding ratio for that segment. A copy of the full program is available from the authors.

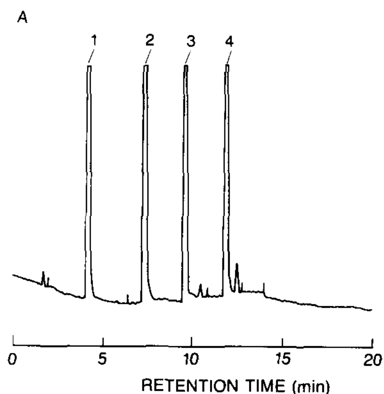
When the possible internal standards in the user-specified retention Window are displayed, their adjusted retention times are also shown. Other database files could be created and used with Window, including databases for different solvents gradients or isocratic conditions.

Internal standard selection

An aliquot of 2 μl of the marker solution is injected and chromatographed as described above and 2 μl of an appropriate concentration of the sample for which an internal standard is desired are alo injected. The concentration should be sufficient to show the impurities. The use of a sample enriched in impurities might be helpful. A larger injection volume and the use of a more sensitive monitoring wavelength (210 nm) could be used. The four-marker compound retention times are entered into the Window program, which adjusts retention times in the database to the particular column being used.

The sample chromatogram is examined for potential internal standard locations. Because gradient elution will tend to give a more compressed chromatogram than isocratic elution, this must be taken into account when the internal standard is being selected for an isocratic application. The retention window of interest is entered into Window (when prompted) for the window "start" and "end" times. The program will display a sorted list of possible internal standards. Window will ask if the user wants to specify another window or quit.

An example for the internal standard selection method is given in Fig. 2. The marker solution is injected in the analyst's column, Partisil 5 ODS-3, and chromatographed under the prescribed conditions (Fig. 2A). The marker solution retention times are entered into the computer to rearrange the database. Then, an impure insecticide is injected and examined under the same conditions except the separation was monitored at 210 nm to accentuate the presence of the impurities (Fig. 2B). Following examination of the insecticide chromatogram for possible "open windows", the computer was requested to suggest internal standards for these retention time intervals. The computer suggested hexamethylenebenzene (13.4 min) and 1,4-dibromonaphthalene (13.6 min). The latter was selected because of its better spectral



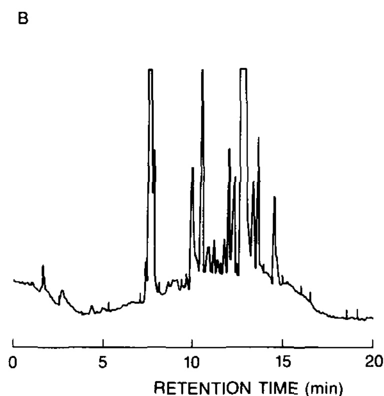


Fig. 2. Liquid chromatograms of marker solution and impure insecticide. (A) marker solution containing components: 1 = acetanilide, 2 = α-tetralone, 3 = benzophenone and 4 = *trans*-stilbene as chromatographed on Partisil 5 ODS-3, 150 mm × 4.6 mm I.D., with a linear gradient from 20 to 100% B in 15 min, where A = 0.005 *M* aq. H_2SO_4 and B = acetonitrile. The flow-rate was 1 ml/min with detection at 254 nm for an injection volume of 2 μl. Signal range was 0.064 a.u.f.s. (B) An impure insecticide chromatographed under the same conditions, except detection was at 210 nm.

compatibility. Spectra for the individual internal standards are available for examination to assess their suitability for absorption at a certain wavelength. These spectra are available from the authors.

DISCUSSION

Aqueous acetonitrile eluents are generally preferred over aqueous tetrahydrofuran and aqueous methanol for reversed-phase chromatography, because they are more UV-transparent and produce less backpressure. Therefore, our internal standard selection approach was centered on aqueous acetonitrile. There are differences in selectivity when the retention time for the latter two mobile phases are compared with

TABLE I

REVERSED-PHASE LIQUID CHROMATOGRAPHIC RETENTION TIMES BY GRADIENT ELUTION WITH THE RESPECTIVE SOLVENTS IN THE "B" RESERVOIR

Compound	Retention time (min)		
	Acetonitrile	Methanol	Tetrahydrofuran
Acetic acid amide (N-4-hydroxyphenyl)	1.40	2.23	1.51
Acetaminophen	1.40	2.24	1.51
Saccharin	1.59	2.48	1.70
2,6-Dimethyl-4-pyrone	1.94	3.72	1.11
Benzamide	1.96	3.42	1.69
2-Acetamodiphenol	2.20	3.55	2.51
Phenylurea	2.20	3.77	2.31
Benzoic acid, 2-methylamino	2.32	4.09	1.91
Salicylamide	2.79	4.63	3.33
4-Hydroxyacetophenone	2.79	4.88	3.22
Phthalimide	2.84	4.87	2.92
Phthalic acid diamide	2.84	4.87	2.95
Acetanilide	2.99	4.91	2.74
4-Methoxyphenol	3.21	4.70	3.73
2-Methoxybenzoic acid	3.27	5.68	2.78
Benzoic acid	3.86	6.88	4.58
4-Methoxybenzoic acid	4.64	7.34	4.18
4,4′-Biphenol	4.50	7.05	5.50
Coumarin	4.53	6.58	3.45
1,1-Diphenylurea	4.87	8.30	3.28
o-Toluic acid	4.88	8.36	5.52
m-Toluic acid	5.04	8.70	5.42
Tri(4-hydroxyphenyl)methane	5.23	8.11	5.33
Acetophenone	5.14	7.16	4.12
Methane bis(4-hydroxyphenyl)	5.33	8.61	5.11
Acetamide 2,4-dichlorophenoxy	5.80	9.55	5.28
3,5-Dimethylphenol	5.95	8.80	3.46
4-Bromophenol	6.02	8.84	6.22
4-Ethylphenol	6.14	8.91	6.27
Benzamide: 3-methyl-N,N-diethyl	6.48	9.74	3.86
α-Tetralone	6.40	9.02	4.05
4-Hydroxybenzoic acid, methyl ester	6.52	9.83	6.38
Bisphenol A	6.58	10.03	5.87
trans-4-Phenylcyclohexanol	6.72	10.74	6.87
2,4-Dichlorophenoxyacetic acid	6.76	10.11	6.87
4-Methoxybenzoic acid	6.79	9.61	5.43
4-Methoxypropiophenone	6.86	9.44	4.64
Propiophenone	6.88	9.06	5.68
1,3-Diphenylurea	6.93	9.47	6.82
4-Chloroacetophenone	7.09	9.54	5.96
2,4-Dihydroxybenzophenone	7.16	10.41	6.96
Diphenylsulfone	7.20	8.61	5.37
n-Butoxyphenol	7.38	10.32	6.13
4-Phenylphenol	7.55	10.73	6.11

(Continued on p. 204)

TABLE I *(continued)*

Compound	Retention time (min)		
	Acetonitrile	Methanol	Tetrahydrofuran
Benzoic acid 2-hydroxyamino-4-phenyl	7.82	10.21	6.10
4-*tert.*-Butylphenol	7.94	11.05	7.02
2-Phenylphenol	7.90	10.67	7.16
4-Benzylphenol	8.04	11.17	7.20
n-Butyrophenone	8.30	10.53	6.07
Benzophenone	8.85	11.19	6.84
9-Fluorenone	8.92	11.61	5.81
4-Phenylacetophenone	9.29	12.14	6.67
1,3-Diphenyl-2-propanone	9.22	11.31	7.42
Benzil	9.18	11.27	7.07
Diphenyl carbonate	9.31	11.62	5.41
2,4,6-Tribromophenol	9.45	12.73	8.25
Valerophenone	9.54	11.03	7.61
4-Cyclohexylphenol	9.64	12.76	7.95
Naphthalene	9.64	12.27	7.63
2-Hydroxybenzoic acid, phenyl ester	10.07	12.83	5.95
Triphenylmethanol	10.27	12.86	6.92
Chlorotriphenylmethane	10.28	12.86	6.85
Diphenyl oxide	10.59	13.03	7.98
Biphenyl	10.73	13.38	8.20
Hexanophenone	10.76	12.92	8.25
Dibenzofuran	10.80	13.58	7.94
2-Methylnaphthalene	10.90	13.50	8.19
Fluorene	11.20	13.99	8.29
Dibenzo-*p*-dioxin	11.30	14.06	
2-Bromonaphthalene	11.48	13.84	8.34
1-Bromonaphthalene	11.53	13.90	8.16
Benzylbutylphthalate	11.54	13.53	8.42
trans-Stilbene	11.75	14.33	8.34
4-Phenyltoluene	11.78	14.34	8.70
2-Ethylnaphthalene	11.83	14.21	8.69
Dibutylphthalate	11.86	13.62	8.48
1,2-Diphenylethane	11.90	14.38	8.01
Butylbenzene	weak	weak	weak
4,4′-Ditolylether	12.52	14.68	9.03
m-Diphenoxybenzene	12.86	14.85	9.04
Triphenylmethane	12.77	14.83	
Octanophenone	12.92	14.50	9.21
m-Diphenoxybenzene	12.87	14.85	9.04
2,6-Di-*tert.*-butyl-4-methylphenol	13.43	14.87	9.88
Hexamethylenebenzene	13.55	15.77	9.72
1,4-Dibromonaphthalene	13.77	15.38	8.93
Tetraphenylethylene	14.51	15.70	9.52
Decanophenone	14.78	15.57	9.91
Heptylbenzene	15.14	16.04	8.37
Laurophenone	16.14	16.35	10.51
Dioctylphthalate	16.74	16.50	10.70
Myristylphenone	17.36	17.07	10.98

aqueous acetonitrile, as shown in Table I. The general approach should still be applicable, but there would be less certainty in the selection process.

The 90 potential internal standards, together with the marker solution, were chromatographed by 0.005 *M* sulfuric acid–acetonitrile gradient elution on three additional reversed-phased octadecyl columns. Using the marker solution retention times from the Spherisorb, Partisil and Zorbax columns and the database from the Nova-Pak column, the retention times for the 90 potential internal standards were obtained from the computer for the three columns. When compared with the actual retention times measured for these columns, the average difference was 0.02 min with an average standard deviation of 0.34 min.

The use of multicomponents in the marker solution was expected to provide a more satisfactory correction of retention times, should a manufacturer's column show more variability towards polar and/or non-polar compounds. Also, this would tend to average out retention time corrections, should there be some column selectivity towards a particular class of compounds.

CONCLUSIONS

A four-component marker solution and the sample for which an internal standard is desired is injected into any reversed-phase column of the analyst's choice and developed under prescribed gradient conditions. After entering the retention times for the marker solution, the sample chromatogram is examined for "open windows". The computer is then requested to suggest internal standards for these time windows.

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