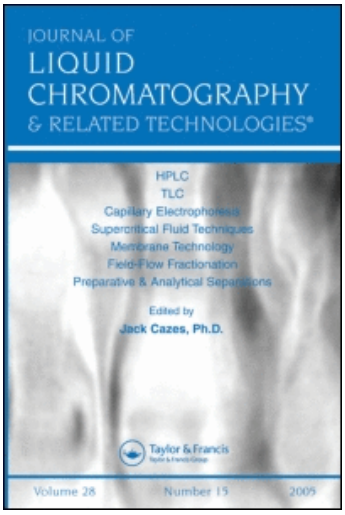

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APPLICATION OF NORMAL AND SECOND-ORDER DERIVATIVE SPECTROSCOPY IN IDENTIFYING ORGANIC ACIDS AND SUGAR ACIDS IN LIQUID CHROMATOGRAPHY WITH ON-LINE PHOTODIODE ARRAY DETECTION

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ABSTRACT

The UV-spectra of 16 compounds including organic acids and sugar acids were recorded at pH 2.5 using photodiode array detection in three different chromatographic systems: an ion exchange column with phosphate buffer as eluent (I), a C-8 reversed-phase column with sulphuric acid as eluent (II) and a C-8 reversed-phase column with hydrochloric acid as eluent (III). Differences in the normal spectra in each of the systems were evaluated by calculating match factors between the spectra. Normal spectra and the second-order derivative spectra gave match factor ranges of 603 - 1000 and 0.1 - 999, respectively. The similarity between the normal spectra was very good and excellent between chromatographic systems I and II. On the other hand, the similarity between the second-order derivatives of the spectra was not as good. Thus the normal UV-spectra can be used, together with the retention times, for reliable identification despite of the spectral similarity of some acids. Molecular connectivity indices up to the sixth order were correlated with the retention parameters. The correlation was only moderate in all the systems.

INTRODUCTION

The development of diode-array detectors has been the most important advance in HPLC for the quantitative identification of separated compounds. During the past few years many significant improvements have been made in software and in the engineering of detectors capable of achieving better wavelength resolution and sensitivity (1). With a diode array detector, spectra can be acquired automatically at the peak apex as each peak elutes. The spectra can be compared with those stored in a library (2,3). Such libraries are unfortunately not commercially available and have to be built up by the individual laboratory.

In the case of chemical compounds only those with double bonds and/or phenyl rings usually exhibit UV absorbance. The greater the number of chromophores in the compound the more useful is the UV spectrum for identifying the compound. For example, polycyclic aromatic hydrocarbons (PAH) are very outstanding because of their wide and characteristic UV spectra (2). Self-made UV spectra libraries have been used successfully for identifying many kinds of organic compounds (3,4).

Derivative spectra enhance the differences between spectra by revealing subtle changes in slope that are difficult to observe in the normal spectra (5,6). Second-order derivatives reveal more specific details of the spectra. Peptides especially, show spectral differences in their derivative spectra [2]. However, derivative spectra must be handled with care. Both the standard and unknown spectra should be noise free.

Organic acids are extraordinarily difficult to identify on the basis of their UV spectra because their only chromophore is the carboxyl moiety. The aim of the study was to identify and estimate limitations and potentials of diode array

detection and library matching in identifying organic acids and sugar acids using normal and second derivative spectra. The retention of the compounds was also correlated with the molecular connectivity indices (7-9) in order to determine whether the retentions can be predicted in the systems used.

MATERIALS AND METHODS

Instrumentation

A Hewlett-Packard model 1090 HPLC equipped with an HP 1040A diode array detector was used. An HP data station was used for data storage, comparison and mathematical manipulation of the acquired spectra.

Columns and Chromatographic Procedures

System I: The column was a Bio-Rad Organic Resolution column 30 cm * 8 mm, I.D. The mobile phase was prepared by mixing 0.125 M KH_2PO_4 and 0.125 M K_2HPO_4 (1:1), and then adjusting the pH with phosphoric acid to 2.5. The flow-rate was 0.8 ml/min and the oven temperature 40 C.

System II: A 20 cm * 4.6 mm I.D. column was packed under 500 bar pressure with LiChrosorb C_8 (Merck, Darmstadt), using a slurry technique with acetone as the suspending medium and a 50 ml slurry reservoir. The mobile phase was prepared by adding sulphuric acid into distilled water until the pH was 2.5. The flow-rate was 1 ml/min and the oven temperature was 40 C.

System III: Same column as in system II. The mobile phase was prepared by adding hydrochloric acid into distilled water until the pH was 2.5. The flow-rate was 1 ml/min and the oven temperature was 40 C.

Reagents

Citric acid, maleic acid, succinic acid, formic acid, acetic acid, sulphuric acid, phosphoric acid, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). Malic acid , malonic acid, tartaric acid, propionic acid , butyric acid, galactonic acid - lactone, glucuronic acid, gluconic acid and gulonic acid - lactone were from Fluka (Buchs, Schwitzerland). Lactic acid was from K&K Laboratories (Cleveland, Ohio). Galacturonic acid was from California corporation for Biochemical Research (Los Angeles, USA). Hydrochloric acid was from Carlo Erba (Milano, Italy). Acetonitrile was of HPLC grade (Merck) and the water was distilled and deionised.

Calculation of the Molecular Connectivity Indices

The molecular connectivity indices for the acids were calculated using the Molconn-X programme (Hall Associates Consulting, Massachusetts, USA) developed by L.H. Hall.

RESULTS AND DISCUSSION

To confirm the identity of a UV spectrum the spectrum must be compared with a set of standard spectra from a spectral library (2). A visual comparison of these spectra usually takes a long time and is not suitable for automated operations. The correlation coefficient for all the corresponding absorbances measured gives the best results (10). With the HP instrument the absorbances can be measured at intervals of 2 nm. Comparison of two spectra is thus based on the correlation coefficient and gives the match factor (coefficient * 1000, range from 0 to 1000). A match of 0 indicates there is no match and 1000 indicates identical spectra. Values greater than 990 generally indicate the spectra are similar. Values between 900-990 indicate there is some similarity, and values below 900 indicate the spectra are different.

The spectra of the organic acids were recorded in three different chromatographic systems. The spectra were recorded at concentrations of the acids that gave maximum absorption in each spectrum stored in a library of 200-800 mAU. The pH was adjusted to a rather low value (2.5) because organic acids have to be analysed liquid chromatographically at a low pH to ensure that the compounds are not in an ionised form. The spectra were recorded from 200 nm to 350 nm even though the only chromophore in the compounds (apart from maleic acid) is the carboxyl moiety which only show absorption at about 200-250 nm in the low UV region.

Repeatability of the Recording Spectra

The repeatability of the recording spectra was tested by first recording the spectrum of each of the compound in each of the systems and then repeating the same procedure. The match factor between the first and second measurement was at least 990.

Differences in Spectra in a Given Chromatographic System

In order to reveal the differences in spectra in a given chromatographic system the UV spectra of the 16 compounds were compared with each other in each of the systems. Thus a total of 120 comparisons was made in each system. Normal spectra had a match factor of 683 - 1000 in system I, 673 - 999 in system II and 603 - 999 in system III. Correspondingly the second-order derivative spectra had a match factor range of 12 - 999 in system I, 0.1 - 995 in system II and 0.1 - 995 in system III. The match factors for each of the acids were compared to all others and the factors thus obtained were averaged in the three systems. The averaged match factors are presented in Table I. The lower the mean match factor is, the more the spectra differ from the spectra of the other acids investigated. Comparison of some of the recorded spectra and their second-order derivatives are presented in Figure 1. The differences in spectra show that, although the carboxyl

TABLE 1

Mean Match Factors for the Normal and Second-Order Derivative Spectra of Each of The Acids Compared to Other Acids in The Three Systems.

Compound	Normal spectra System			Second-order spectra System		
	I	II	III	I	II	III
Lactic acid	926	921	822	794	424	185
Citric acid	916	933	949	833	797	761
Malic acid	920	940	952	844	803	770
Malonic acid	944	956	961	787	735	733
Maleic acid	950	964	947	726	716	670
Succinic acid	928	934	944	679	683	623
Tartaric acid	923	939	952	786	771	721
Formic acid	923	939	952	790	763	743
Acetic acid	928	933	941	618	627	551
Propionic acid	926	934	943	702	704	656
Butyric acid	952	964	944	753	750	721
Galactonic acid -lactone	831	838	791	324	229	222
Glucuronic acid	934	937	946	714	677	677
Gluconic acid	906	906	960	585	528	722
Gulonic acid -lactone	841	846	869	457	424	386
Galacturonic acid	927	927	934	743	717	641

moiety is the only chromophore, the other part of the molecule also affects the spectrum. On the other hand, in many cases the spectrum of a specific acid was almost identical to 1-5 of the other spectra. However, the acids can usually be separated very easily chromatographically and the spectrum is only recorded in order to verify the identity of the compound.

The second order-derivative spectra show greater differences in spectra than the normal spectra (Table I). It could be concluded that second-order derivative spectra are useful in identifying the compounds. However, because the acids absorb

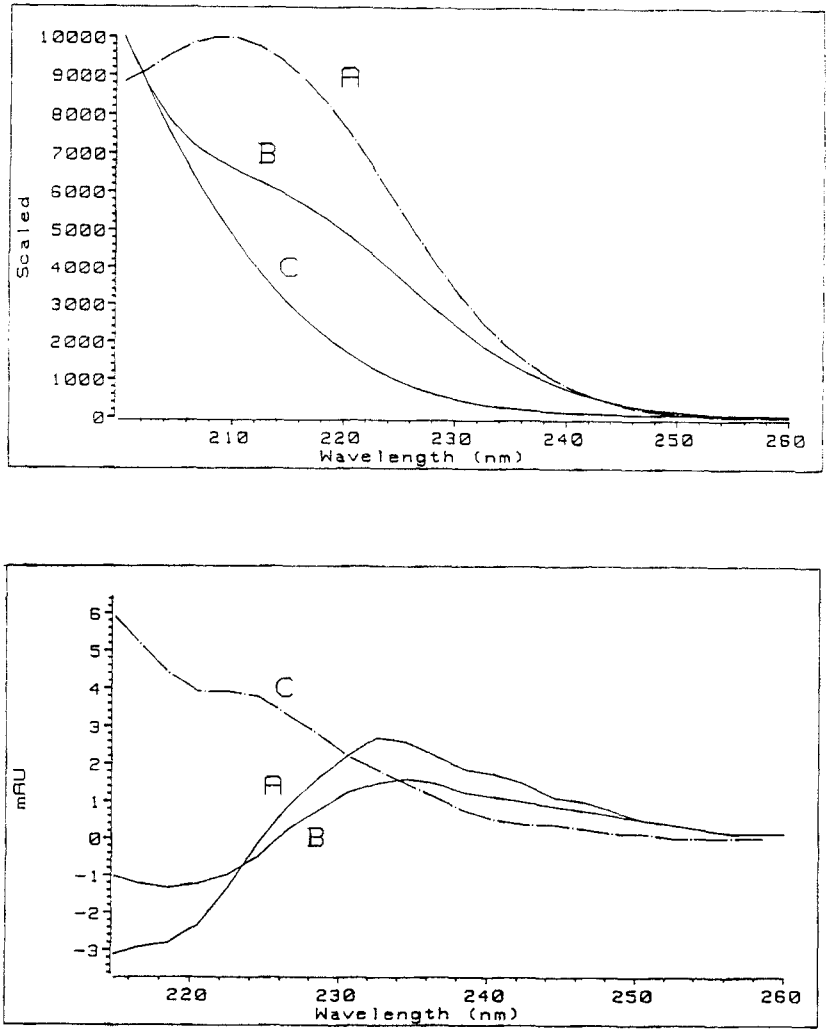

FIGURE 1. Normal spectra (upper) and second order derivative spectra (lower) of citric acid (A), glucuronic acid (B) and lactic acid (C) in chromatographic system I. The match factors in the normal and in the second-order derivative spectra are as follows: A-B: 906, B-C: 857, A-C: 684 and A-B: 941, B-C: 401, A-C: 882, respectively.

TABLE 2

Mean Match Factors for the Normal and Second-Order Derivative Spectra of a Particular Acid Compared to the Same Acid in Another Chromatographic System.

Compound	Normal spectra System			Second-order spectra System		
	I-II	I-III	II-III	I-II	I-III	II-III
Lactic acid	1000	994	995	980	353	369
Citric acid	996	984	993	904	680	855
Malic acid	998	984	996	939	652	775
Malonic acid	993	987	989	966	642	631
Maleic acid	994	997	993	996	632	567
Succinic acid	1000	993	995	995	61	466
Tartaric acid	996	982	997	968	729	767
Formic acid	1000	989	994	991	670	689
Acetic acid	1000	994	994	842	8	178
Propionic acid	1000	993	993	474	691	890
Butyric acid	1000	995	992	994	433	451
Galactonic acid -lactone	996	972	978	769	859	926
Glucuronic acid	998	999	999	942	713	697
Gluconic acid	990	980	980	47	590	614
Gulonic acid -lactone	999	967	978	967	918	948
Galacturonic acid	998	996	998	973	13	348
Mean	997	988	993	859	540	636
S.D.	3	9	7	254	288	226

in the low UV region (200-250 nm), where disturbances caused by the eluent are strongest, and the disturbances multiplies when taking the derivative spectrum, the second-order derivative spectra are not very useful in identifying such compounds.

Influence of the Chromatographic System on the Spectra

As mentioned earlier the spectra obtained with a specific system can be recorded repeatedly. When the main absorption of a compound occurs at a higher

wavelength (> 250 nm), the disturbances caused by the eluent are minimal and the prediction of compound structure by a UV-spectra library search is very reliable. However, in the present study where the only absorbtion occurred in the relatively low UV-region (< 250 nm), the chromatographic system had an effect on the spectra.

The mean differences in the match factors between the spectra obtained in the three different chromatographic systems are presented in Table II. The similarity between the normal spectra is very good and excellent between chromatographic systems I and II. On the other hand, the correspondence between the second-order derivatives of the spectra is not nearly as good. This is probably due to absorption by the ions in the eluent. Thus the second-order spectra are not useful for identifying these acids if the eluent composition is changed.

Comparison of log k' Values with the Molecular Connectivity Indices

The molecular connectivity indices were calculated for the compounds given in Table I. Some of the compounds contained so few atoms that the valence and connectivity indices could be calculated only to the second order. The SAS RSQUARE procedure (11), which performs all possible regressions for dependent variables, and ranks them according to correlation coefficient, was run to obtain regressions of log k' against all two variable combinations of the indices. The best regression equations for the systems I-III are given below:

System I:

$$\log k' = 0.247\ {}^{0}\chi^{v} - 0.296\ {}^{1}\chi + 0.797 \qquad r=0.758 \quad (1)$$

System II:

$$\log k' = 0.514\ {}^{0}\chi^{v} - 0.590\ {}^{1}\chi + 0.0898 \qquad r=0.703 \quad (2)$$

System III:

$$\log k' = 0.473\ {}^{0}\chi^{v} - 0.541\ {}^{1}\chi + 0.0537 \qquad r=0.734 \quad (3)$$

The moderate correlation coefficients indicate that the prediction of retention on the basis of the molecular connectivity indices is not reliable, probably as a result of the relatively large number of oxygen atoms in the compounds studied. Prediction of the retention of oxygen-containing compounds using the molecular connectivity indices on reversed-phase columns has been shown to be difficult (12,13).

CONCLUSIONS

Organic acids are extremely difficult compounds to identify on the basis of their UV spectra owing to the great similarity of their spectra. However, they can be easily separated chromatographically and the UV spectra, together with the retention data, can be used for rather reliable identification. Because of the disturbances in the low UV region (< 250 nm), second order derivative spectra are not useful for identification.

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