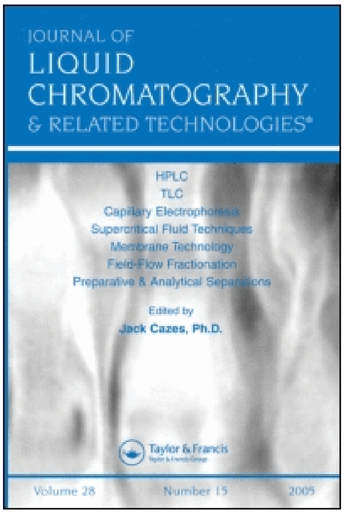

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# A Computer Program for the Identification of the Elution Order of Peaks in High Performance Liquid Chromatography

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# A COMPUTER PROGRAM FOR THE IDENTIFICATION OF THE ELUTION ORDER OF PEAKS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1]

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## INTRODUCTION

High performance liquid chromatography (HPLC) is a rapidly growing separation technique. Its popularity is due to its relatively low cost, selectivity, ease of operation and the ability to separate different compounds (structurally and chemically), of large and small molecular size. The results can be quantified quickly and easily.

The most difficult aspect of HPLC is the selection of a mobile phase that gives good resolution of the components of a mixture in a reasonable time, with optimum separation. Recently, many research papers have suggested a systematic approach to mobile phase selection for the optimum separation of a mixture, by using statistical methods of analysis. Glajch et al (1) and Issaq et al (2) have used such a technique based on the work of Snee (3) which employed overlaping resolution mapping for the selection of a mobile phase that would give optimal separation. To establish such a mobile phase, the analyst should run 7-10 experiments using different solvent combinations of three

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[1] Presented in part at the Drug Metabolism Discussion Group Meeting, Plymouth Meeting, PA, May 6, 1982.

TABLE 1

RATIO OF SOLVENT COMBINATIONS USED

| Experiment # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| % Solvent A | 100 | 0 | 0 | 50 | 50 | 0 | 33 | 20 | 20 | 60 |
| % Solvent B | 0 | 100 | 0 | 50 | 0 | 50 | 33 | 60 | 20 | 20 |
| % Solvent C | 0 | 0 | 100 | 0 | 50 | 50 | 33 | 20 | 60 | 20 |

Note: Other solvent combinations may be used; the analyst needs 10 data points. The ratios can be different from those above.

organic solvents (Table 1). The solvents selected, based on Synder's solvent Synder's solvent selectivity triangle (4), should have different chemical properties in order to give different selectivities of the solutes. Belinky (5), on the other hand, used 17 combinations of three solvents to achieve an optimum mobile phase. Laub (6) used the window technique, which was developed for the selection of GC mixed phases, and which would require a minimum of 10 experiments to achieve an optimum mobile phase if three different solvents were used. Others (7-9) have used statistical approaches which in the end led to an isocratic mobile phase that efficiently separated the components of the mixture under study.

Finding a mobile phase that would separate the components of a mixture using statistical, or any other, technique can be time consuming because, in each of the experiments, solvents of different selectivities are used, and this can lead to a different elution orders of the components, (ref. 2 gives a good example). This means that the analyst would have to identify each eluted peak at the end of every experiment. Assuming that a mixture is composed of six components, and that they elute off the column in each of the 10 required experiments in an average of 15 min, it would take a chromatographer approximately 12-15 hours or 2 days to identity the peaks in all 10 experiments.

DuPont (10) have developed the Sentinel System whereby eluted peaks are identified, if there is no peak crossovers, by a statistical technique. The company has not released this information. If peak crossover takes place, standard solutions should be injected and elution times compared to identify the composition of the peaks. In this case, the analyst needs to know if peak crossover takes place, which is difficult to ascertain unless standards are injected and eluted. James (11) described wavelength chromatography for the identification of eluted components, which is based on a multiwavelength monitoring system employing a diode array spectrophotometer. This is undoubtedly an accurate and rapid method, which also requires the use of a computer, and that the solutes should absorb light in the 200-800nm range.

We present here a computer program (Appendix 1) which can identify the peak's elution order, taking into consideration (a) peak reversal; (b) peaks coalescing, i.e. six peaks in one run but five peaks or less in another; and (c) peak splitting, i.e. four peaks in one mobile phase and six in another. The peak elution order identification is based on one final run of the standards. The peaks are identified by the area ratio of each peak compared with the other eluted peaks in that experiment. It is assumed that the peaks are Gaussion and symmetrical, with no peak frontage or tailing. Since absorption is a function of the number of molecules present, the shape of the peak due to diffusion should not affect the area under the peaks. In HPLC the peak which elutes first is sharper than it would be if it eluted last because of diffusion. It is also possible that the extinction coefficient of a compound can change from one solvent to another. Since mixtures of solvents are used this phenomenon is minimized (Table 2).

This computer program is also suited for use with radiolabeled compounds and their metabolites, since the number of counts is directly proportional to the amount of radioactivity. A selected hypothetical example will be presented which illustrates the value of this computer program.

TABLE 2

Effect of Mobile Phase Composition on the % Peak Areas of Anthraquinone (A), Methylanthraquinone (MA), and Ethylanthraquinone (EA)

| | A (%) | MA (%) | EA (%) |
|---|---|---|---|
| $CH_3CN:H_2O$ / 64:36 | 22.5 | 40.6 | 36.9 |
| $CH_3OH:H_2O$ / 75:25 | 22.2 | 41.1 | 36.7 |
| $THF:H_2O$ / 45:55 | 23.6 | 40.7 | 35.7 |
| $CH_3OH:CH_3CN:H_2O$ / 38:32:30 | 22.4 | 40.8 | 36.7 |
| $CH_3OH:THF:H_2O$ / 38:22:40 | 22.8 | 41.0 | 36.2 |
| $CH_3CN:THF:H_2O$ / 32:22:36 | 22.7 | 40.8 | 36.5 |
| $CH_3OH:CH_3CN:THF:H_2O$ / 25:21:15:39 | 23.3 | 40.7 | 36.0 |
| $CH_3CN:THF:CH_3OH:H_2O$ / 42:7.5:12.5:38 | 23.2 | 40.0 | 36.8 |
| $CH_3CN:THF:CH_3OH:H_2O$ / 11:30:12.5:46.5 | 23.4 | 41.0 | 35.6 |
| $CH_3CN:THF:CH_3OH:H_2O$ / 11:7.5:50:31.5 | 23.1 | 40.6 | 36.3 |
| **Mean ± Standard Deviation:** | 22.9 ± 0.5 | 40.7 ± 0.3 | 36.3 ± 0.5 |

## EXPERIMENTAL

Materials: Solvents were glass distilled (Burdick and Jackson). Chemicals were analytical grade (Aldrich Chemical Co.) and used without further purification.

Apparatus: A modular HPLC system consisting of Laboratory Data Control (LDC) Constametric I and II pumps attached to an LDC Gradient Master, a Chromatronix dual-channel uv absorbance detector, Rheodyne injector, and a strip-chart recorder operated at 0.2 in/min. was used.

The RP-18 reversed phase column (Merck) was 250 mm x 4.6 mm prepacked with 10 μm particle size materials. 10 μl samples were injected. Experiments were run at room temperature using a mobile phase flow rate of 1.2 ml/min. Retention times, peak areas, and peak area percent were determined with a Hewlett-Packard

1865 A/D converter connected to the UV detector output of the liquid chromatograph. The output from the data system was recorded on a 9866A thermal line printer (Hewlett-Packard).

## COMPUTER PROGRAM

The PKSEP program which identifies peaks by peak area percent is written in Basic for a Hewlett Packard 3354 Lab Data System. This system uses a model 2100 CPU with 32 K of core. Currently, the program allows for as many as 12 runs with up to 15 peaks in each run, storing retention times, peak area percents and results of the analysis for each run.

Eluted peaks are identified by comparing the peak area percents of the trial run peaks to those of a standard run. Peak area percents are computed automatically by the HP 3354 Lab Data System. Currently the retention times and area percent for each peak are entered in the program, but the program can be modified to accept the retention time and area percent from the processed data files of each LC analysis.

The run with the most peaks is chosen as the standard. If there are several runs to choose from, the last one entered is used unless the user selects another. The selected run must have the maximum number of peaks.

The analysis for each trial run is as follows. For each trial peak, the standard peak or sum of two peaks which best match the trial peak area percent is chosen. Peak summing is not used at this point if the trial run has the same number of peaks as the standard run. If a standard peak is chosen which defined a previous peak, both trial peak definitions are rechecked and only the better area percent match is kept. If the trial run has the same number of peaks as the standard, standard peaks are not summed in this initial selection. If the area percent of the standard selected differs from the trial by more than 10%, a warning message is printed. Mis-matches of this size will most probably be resolved in the identification of later trial peaks. This initial selection produces no peak identification conflicts, but all trial and standard peaks may not have been matched. All unused trial peaks are then compared against all unused standards and the best available area percent fit is used, if the areas match by 90% or more. Note that not all standard peaks are forced to match a trial peak due to this 90% requirement. All selected peaks, warning messages and re-defined standards are printed out during the analysis.

A summary report of each trial run in elution order of the standard run is printed out, showing the corresponding trial peak number, retention time and area %, or a message is given to indicate that the standard peak cannot be identified.

Finally a summary table giving the elution order for each run is printed out.

## A HYPOTHETICAL EXAMPLE

A mixture containing seven components was selected as an example. The output is listed as follows:

```
ENTER RT, AREA % FOR EACH PEAK FROM RUN 1
  END WITH -1,-1
:? 1.1,6%
:? 2.7,4%
:? 11,20%
:? 21.4,15%
:? 23.7,14%
:? 34,21%
:? 37,19%
? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 2
  END WITH -1,-1
:? 1.1,20%
:? 11.2,30%
:? 22.3,10%
:? 35.7,40%
:? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 3
  END WITH -1,-1
:? 1,10%
:? 12,19.5%
:? 23.2,14.9%
:? 24.7,14.1%
:? 35.7,40%
:? -1,-1
```

```
ENTER RT, AREA % FOR EACH PEAK FROM RUN 4
  END WITH -1,-1
:? 1,5.9%
:? 2.4,4.1%
:? 11.3,30.1%
:? 23.1,14.8%
:? 27.3,14.1%
:? 35.2,20.9%
:? 37.8,19.1%
:? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 5
  END WITH -1,-1
:? 1,5.8%
:? 2.1,4.2%
:? 11.1,20.1%
:? 13.4,14.9%
:? 15.8,14.1%
:? 24.7,21.9%
:? 31.2,18.6%
:? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 6
  END WITH -1,-1
:? 1,48%
:? 2.4,6%
:? 13.2,15%
:? 21.7,14%
:? 25.7,17.4%
:? -1,-1

ENTER RT, AREA % FOR EACH PEAK FROM RUN 7
  END WITH -1,-1
:? 1,10%
:? 2.3,20%
:? 3.5,30%
:? 4.8,40%
:? -1,-1
```

These are the seven required runs to perform an optimum solvent mixture analysis using seven different mobile phases. The next step is the analysis of the areas in these seven runs to determine which area belongs to which peak/ peaks.

Run # 1 is selected as the standard; it has the maximum number of peaks. The computer will identify the peaks in the remaining six runs as follows:

```
ANALYSIS FOR RUN #2
    PEAK #1     - 20     STD #3     - 20
    PEAK #2     - 30     STD #4     - 15     + #5     - 14     = 29
    PEAK #3     - 10     STD #1     - 6      + #2     - 4      = 10
    PEAK #4     - 40     STD #6     - 21     + #7     - 19     = 40
```

```
ANALYSIS FOR RUN #3
    PEAK #1      - 10      STD #1      - 6      + #2      - 4      = 10
    PEAK #2      - 19.5    STD #7      - 19
    PEAK #3      - 14.9    STD #4      - 15
    PEAK #4      - 14.1    STD #5      - 14
    PEAK #5      - 40      STD #6      - 21     + #7      - 19     = 40
   *** REDEFINITION OF STD  7      ***
    PEAK #2       MAY BE  STD #3




ANALYSIS FOR RUN #4
    PEAK #1      - 5.9     STD #1      - 6
    PEAK #2      - 4.1     STD #2      - 4
    PEAK #3      - 30.1    STD #6      - 21
   ***PEAK AREAS DIFFER BY >10% ***
    PEAK #4      - 14.8    STD #4      - 15
    PEAK #5      - 14.1    STD #5      - 14
    PEAK #6      - 20.9    STD #6      - 21
   *** REDEFINITION OF STD  6      ***
    PEAK #7      - 19.1    STD #7      - 19




ANALYSIS FOR RUN #5
    PEAK #1      - 5.8     STD #1      - 6
    PEAK #2      - 4.2     STD #2      - 4
    PEAK #3      - 20.1    STD #3      - 20
    PEAK #4      - 14.9    STD #4      - 15
    PEAK #5      - 14.1    STD #5      - 14
    PEAK #6      - 21.9    STD #6      - 21
    PEAK #7      - 18.6    STD #7      - 19




ANALYSIS FOR RUN #6
    PEAK #1      - 48      STD #3      - 20     + #6      - 21     = 41
   *** PEAK MAY BE SUM OF 3 OR MORE STDS ***
    PEAK #2      - 6       STD #1      - 6
    PEAK #3      - 15      STD #4      - 15
    PEAK #4      - 14      STD #5      - 14
    PEAK #5      - 17.4    STD #2      - 4      + #5      - 14     = 18
   *** REDEFINITION OF STD  5      ***
    PEAK #5       MAY BE  STD #7




ANALYSIS FOR RUN #7
    PEAK #1      - 10      STD #1      - 6      + #2      - 4      = 10
    PEAK #2      - 20      STD #3      - 20
    PEAK #3      - 30      STD #4      - 15     + #5      - 14     = 29
    PEAK #4      - 40      STD #6      - 21     + #7      - 19     = 40
```

```
SUMMARY OF RESULTS


RUN # 2
STD PEAK #      PEAK #          RT          STD AREA %       PEAK AREA %
1      -1.1         3          11           6                10
2      -2.7         3          11           4                10
3      -11          1          1.1          20               20
4      -21.4        2          2.7          15               30
5      -23.7        2          2.7          14               30
6      -34          4          21.4         21               40
7      -37          4          21.4         19               40


RUN # 3
STD PEAK #      PEAK #          RT          STD AREA %       PEAK AREA %
1      -1.1         1          1.1          6                10
2      -2.7         1          1.1          4                10
3      -11          2          2.7          20               19.5
4      -21.4        3          11           15               14.9
5      -23.7        4          21.4         14               14.1
6      -34          5          23.7         21               40
7      -37          5          23.7         19               40


RUN # 4
STD PEAK #      PEAK #          RT          STD AREA %       PEAK AREA %
1      -1.1         1          1.1          6                5.9
2      -2.7         2          2.7          4                4.1
3      -11          - PEAK NOT IDENTIFIED
4      -21.4        4          21.4         15               14.8
5      -23.7        5          23.7         14               14.1
6      -34          6          34           21               20.9
7      -37          7          37           19               19.1


RUN # 5
STD PEAK #      PEAK #          RT          STD AREA %       PEAK AREA %
1      -1.1         1          1.1          6                5.8
2      -2.7         2          2.7          4                4.2
3      -11          3          11           20               20.1
4      -21.4        4          21.4         15               14.9
5      -23.7        5          23.7         14               14.1
6      -34          6          34           21               21.9
7      -37          7          37           19               18.6


RUN # 6
STD PEAK #      PEAK #          RT          STD AREA %       PEAK AREA %
1      -1.1         2          2.7          6                6
2      -2.7         - PEAK NOT IDENTIFIED
3      -11          1          1.1          20               48
4      -21.4        3          11           15               15
5      -23.7        4          21.4         14               14
6      -34          1          1.1          21               48
7      -37          5          23.7         19               17.4
```

```
RUN # 7
STD PEAK #     PEAK #         RT          STD AREA %      PEAK AREA %
1       -1.1        1        1.1          6              10
2       -2.7        1        1.1          4              10
3       -11         2        2.7          20             20
4       -21.4       3        11           15             30
5       -23.7       3        11           14             30
6       -34         4        21.4         21             40
7       -37         4        21.4         19             40
```

Note that in the analysis of run #3, standard peak 7 was selected as a match to peak 2, then selected again as matching peak 5. Standard peak 3 was not initially selected as matching any trial peak, but was matched to peak 2 when testing all unidentified peaks. However in run #4, standard peak 6 was matched first to peak 3, then to peak 6, and standard, and trial, peak 3 are left unidentified even after testing all unmatched peaks, and is recorded as -1, which means that that peak has not been identified. In this case, the chromatographer will have to use other means to identify that peak.

Currently the program sums only two standard peaks if summing is required. The program can be modified to search for the sum of more than two peaks. If the summed area percent is 10% less than the trial peak area percent, a message such as the one in run #6 is printed, indicating that three or more standard peaks may be represented in the trial peak area.

Finally, the computer will list the summary of the above analysis, in table form, (Table 3).

TABLE 3

```
                          RUN #
STD #   1     2     3     4     5     6     7     8     9     10

1       1     3     1     1     1     2     1
2       2     3     1     2     2     -1    1
3       3     1     2     -1    3     1     2
4       4     2     3     4     4     3     3
5       5     2     4     5     5     4     3
6       6     4     5     6     6     1     4
7       7     4     5     7     7     5     4
BASIC
>
```

## CONCLUSION

A computer program has been written which can identify the peak elution order according to their area percent.

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## APPENDIX - Program Listings

```
10  DIM A[180],T[180],P[180],N[12]
20  DEF FNZ(X)=INT(100*X+.5)/100
30  PRINT
40  PRINT "PEAK IDENTIFICATION BY AREA PROGRAM - 4/82"
50  PRINT
60    FOR I=1 TO 180
70    LET P[I]=-1
80    NEXT I
90  LET M=0
100  LET I=1
110  LET N1=0
120  LET T2=0
130  PRINT
140  PRINT "ENTER RT, AREA % FOR EACH PEAK FROM RUN ";I
150  PRINT "  END WITH -1,-1"
160    FOR J=1 TO 15
170    PRINT ":";
180    INPUT T1,A1
185    LET K=(I-1)*15+J
190    IF T1+A1<0 THEN 290
210    IF T1>T2 THEN 240
220    PRINT "PEAKS OUT OF ORDER"
230    GOTO 110
240    LET T[K]=T1
250    LET T2=T1
260    LET A[K]=A1
270    LET N1=N1+1
280    NEXT J
290  IF N1<M THEN 320
300  LET M=N1
310  LET I1=I
320  IF N1<1 THEN 360
330  LET N[I]=N1
340  LET I=I+1
350  IF I<13 THEN  GOTO 110
360  LET N5=I-1
370  PRINT
```

```
380  PRINT "RUN # ";I1;" USED AS STANDARD"
390  PRINT "OKAY";
400  INPUT N$
410  IF N$[1,1]#"N" THEN 440
420  PRINT "RUN # OF STD ";
430  INPUT I1
440  LET N2=N[I1]
450  IF M=N2 THEN 480
460  PRINT "< MAXIMUM # OF PEAKS IN RUN ";I1
470  GOTO 420
>
```

```
480  LET I9=(I1-1)*15
490    FOR K=1 TO N2
500    LET P[I9+K]=K
510    NEXT K
520  LET A[I9+N2+1]=0
530    FOR K=1 TO N5
540    IF K=I1 THEN 850
550    LET K9=(K-1)*15
560    PRINT
570    PRINT "ANALYSIS FOR RUN #";K
580    LET N1=N[K]
590      FOR J=1 TO N1
600      LET M=1000
610      LET A2=A[K9+J]
620        FOR I=1 TO N2
630        LET K8=I+1
640        IF N2=N1 THEN  LET K8=N2+1
650          FOR I2=K8 TO N2+1
660          GOSUB 1620
670          NEXT I2
680        NEXT I
690      LET A8=A[I9+K1]
700      LET A9=A[I9+K2]
710      LET A1=A8+A9
720      PRINT "    PEAK #";J;"- ";A2;TAB(25);
730      IF K2>N2 THEN 770
740      PRINT "STD #";K1;"- ";A8;"+ #";K2;"- ";A9;"= ";A1
750      IF A2>1.1*A1 THEN  PRINT "   *** PEAK MAY BE SUM OF 3 OR MORE STDS ***"
760      GOTO 790
770      PRINT "STD #";K1;"- ";A[I9+K1]
780      IF M/A2>.1 THEN  PRINT "   ***PEAK AREAS DIFFER BY >10% ***"
790      GOSUB 1150
800      IF F>0 THEN 830
810      LET P[K9+K1]=J
820      LET P[K9+K2]=J
830      NEXT J
840    GOSUB 1390
850    NEXT K
>
```

```
860  PRINT
870  PRINT "SUMMARY OF RESULTS"
880    FOR I=1 TO N5
890    IF I=I1 THEN 1010
900    PRINT
910    PRINT "RUN # ";I
920    PRINT "STD PEAK #     PEAK #           RT           STD AREA %   ";
930    PRINT "   PEAK AREA %"
940      FOR J=1 TO N2
950      LET K8=(I-1)*15
960      LET J1=P[K8+J]
970      LET J2=I9+J
980      IF J1<0 THEN  PRINT J;"-";FNZ(T[J2]);TAB(20);"- PEAK NOT IDENTIFIED"
990      IF J1>0 THEN  PRINT J;"-";FNZ(T[J2]);TAB(20);J1,T[J1],A[J2],A[K8+J1]
1000      NEXT J
1010    NEXT I
1020  PRINT
1030  PRINT
1040  PRINT "                        RUN #"
1050  PRINT "STD #  1     2     3     4     5     6     7     8     9    10"
1060    FOR I=1 TO N2
1070    PRINT
1080    PRINT I;
1090      FOR J=1 TO N5
1100      PRINT P[(J-1)*15+I];
1110      NEXT J
1120    NEXT I
1130  STOP
>
```

```
1140  REM **FIND REDEFINED PEAK - WHICH USE IS BETTER FIT
1150  LET F=0
1160  LET K8=N2+1
1170  LET J1=P[K9+K1]
1180  LET I2=K1
1190  IF J1>0 THEN 1240
1200  LET J1=P[K9+K2]
1210  LET I2=K2
1220  IF J1>0 AND K2 <= N2 THEN 1240
1230  RETURN
1240  PRINT "   *** REDEFINITION OF STD  ";I2;"***"
1250    FOR K5=1 TO N2
1260    IF K5=I2 THEN 1280
1270    IF P[K9+K5]=J1 THEN  LET K8=K5
1280    NEXT K5
1290  LET A8=A[I9+I2]
1300  LET A9=A[I9+K8]
1310  LET A1=A8+A9
1320  IF ABS(A1-A[K9+J1])<M THEN 1360
1330  LET P[K9+I2]=-1
1340  LET P[K9+K8]=-1
1350  RETURN
1360  LET F=1
1370  RETURN
>
```

```
 1380  REM **FIND UNDEFINED PEAKS - DO ANY MATCH
 1390    FOR J=1 TO N1
 1400      FOR I=1 TO N2
 1410      IF P[K9+I]=J THEN 1590
 1420      NEXT I
 1430    LET M=1000
 1440    LET A2=A[K9+J]
 1450    LET P[K9+N2+1]=-1
 1460      FOR I=1 TO N2
 1470      IF P[K9+I]>0 THEN 1520
 1480        FOR I2=I+1 TO N2+1
 1490        IF P[K9+I2]>0 THEN 1510
 1500        GOSUB 1620
 1510        NEXT I2
 1520      NEXT I
 1530    IF M/A2>.1 THEN  RETURN
 1540    PRINT "    PEAK #";J;" MAY BE";TAB(25);
 1550    IF K2 <= N2 THEN  PRINT "STD #";K1;" + #";K2
 1560    IF K2>N2 THEN  PRINT "STD #";K1
 1570    LET P[K9+K1]=J
 1580    IF K2<N2+1 THEN  LET P[K9+K2]=J
 1590    NEXT J
 1600  RETURN
 1610  REM **DIFFERENCE IN AREA ROUTINE
 1620  LET A1=A[I9+I]+A[I9+I2]
 1630  LET D=ABS(A1-A2)
 1640  IF D>M THEN  RETURN
 1650  LET M=D
 1660  LET K1=I
 1670  LET K2=I2
 1680  RETURN
 1690  END
>
```