

FMSPILL

(Fluorescence Spillover Correction)

Version 1.2

Users Guide

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INTRODUCTION

FMSPILL mathematically corrects fluorescent intensity data for spillover from adjacent colors. The program corrects data collected using either fixed excitation and emission wavelength pairs or synchronous scanning over a wavelength range. FMSPILL is a companion program to the WINFAC data collection program designed for use with a Perkin-Elmer LS-50/50B spectrofluorimeter. Both programs were developed, and are maintained, by the Fluorescent Microsphere Resource Center (FMRC). If you wish to correct data not collected with the WINFAC program, the file structures expected by FMSPILL for both fixed wavelength and synchronous scan files are presented in Appendix A and B respectively.

To analyze fluorescent intensities gathered through fixed excitation and emission wavelength pair quantification FMSPILL uses a matrix inversion routine to solve for a solution to a simultaneous set of linear equations as described by Schosser, et. al (3). When extracting intensities from a synchronous scan, FMSPILL performs a least squares fit of an over-determined system of linear equations. Similar to the matrix inversion method for fixed wavelengths, the over-determined method describes each unknown by more than one equation Hostetter, et. al (1). FMSPILL utilizes a set of five wavelengths that bound each pure color peak, centered on the maximum intensity and spaced equally down to approximately the half-height location. Schimmel, et. al (2) presents a detailed description of the methods employed by FMSPILL.

To function properly FMSPILL requires that all pure samples precede sample data in the file. The pure samples must be in order of lowest to highest emission wavelength (blue, blue-green, ... scarlet, violet). Pure samples are used to generate a spillover matrix in the case of a fixed wavelength analysis or compute the over-determined matrix in the case of a synchronous scan analysis. If background (pure solvent) values are present in the data file, the program optionally subtracts the background values from the data values prior to mathematical correction. If background subtraction is chosen, the median background values for those background samples collected prior to reading pure samples are subtracted from the pure sample values. The most current background sample is subtracted from subsequent data samples prior to mathematical correction.

HARDWARE REQUIREMENTS

FMSPILL has been tested under the following conditions and we recommend them as a minimum configuration for this program.

- PC fitted with a 486 processor or higher.
- WINDOWS 95 or higher
- 16 Mb RAM

PROGRAM ACQUISITION

FMSPILL is available for download from the web site of the Fluorescent Microsphere Resource Center (<http://fmrc.pulmcc.washington.edu/fmrc/software.shtml>). If you are unable to access the web you may request a copy of the program on diskette by contacting the FMRC staff through any of the following means:

Electronic Mail: glenny@u.washington.edu
Postal Mail: Fluorescent Microsphere Resource Center
University of Washington
Box 356522
Seattle, WA 98195-6522
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GETTING STARTED

When the program is initiated a blank window appears. Use the **File** menu to select which type of analysis you require.

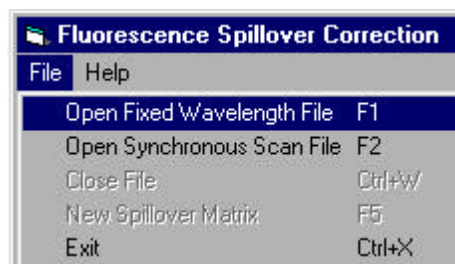
Open Fixed Wavelength File: select to correct data where there is one value for dye in the sample. These files have a .FIX extension.

Open Synchronous Scan File: select to correct data gathered during a synchronous scan where there are multiple points defining a spectrum. These files have a .SYN extension.

FIXED WAVELENGTH ANALYSIS

After choosing the **Open Fixed Wavelength File** menu option the program prompts for three data files:

1. Fixed wavelength data input file (.fix).
2. The next screen prompts for a blank solvent data file (.blk). To perform background subtraction the program must have access to blank samples collected at the time of fluorimetry. If no blank samples were collected press the **Cancel** button to continue.
3. The program then prompts for a Control data file (.ctl). To perform control correction the program must have access to control samples analyzed at the time of fluorimetry. If no control samples are available press the **Cancel** button to continue.
4. Finally the program prompts for the filename of the resultant spillover corrected data. The default filename is the name of the input file with .scff (spillover corrected file) appended to the end.



Following the declaration of all input and output files the program reads through the dataset file(s) to calculate dataset statistics. The cursor spins during this period to assure the user that the program is functioning. The time needed to complete this initial operation is dependent on both the file size and processor speed of your computer. When finished the program displays a listing of dyes/wavelength present and the number of blanks (background pure solvent samples), pures and data samples present (fig 1).

FMSPILL defaults to reporting results in fluorescent intensities. To report results in microspheres/ml. click the appropriate radio button and the colors box resizes to add the microspheres/ml edit boxes (fig 2). The number of wavelengths present in the file is determined from the dataset header. Initially all the colors/wavelengths are selected, but you can deselect those wavelengths you do not wish analyzed. This is useful when the same wavelength/dye has been read using multiple settings. In order for the spillover correction to function properly only one of the multiple readings for a single dye should be selected. If results are requested in microspheres/ml, fill in the number of microspheres/ml used to formulate each of the pure color samples in the corresponding edit box. The procedure for creating pure samples is described in the FMRC manual on regional blood flow determination (4). FMSPILL assumes there is a linear relationship between the number of microspheres and the intensity level detected by the fluorimeter. This holds true as long as the intensity levels are below the quenching threshold for the dye measured (4). The Background Subtraction option is available if a blanks file was selected and was populated.. When background subtraction is selected dye/wavelength intensities from solvent blank samples are subtracted from each data sample prior to mathematical spillover correction. This removes the contribution of the solvent to the sample intensity of each dye. Since the fluorimeter may have temporal electronic drift, the most recently acquired solvent sample intensities are subtracted from each sample, in order to most accurately remove these contributions. Pure samples are used to generate the spillover matrix data so the median of those blanks run just prior to reading the pure samples are used to correct those samples. If a control file is selected and is populated a control correction option is presented. Control correction assumes that any differences between the latest control (standard) reading and the initial control intensities are due to electronic drift of the fluorimeter.



Figure 1: Initial screen for a fixed wavelength analysis. Defaults: (1) results are reported as intensity levels, (2) all wavelengths present are selected, (3) Background Subtraction is selected, and (4) Control Correction is disabled. Background Subtraction and Control Correction are present only if the appropriate files were selected during dataset selection.

When background subtraction is selected

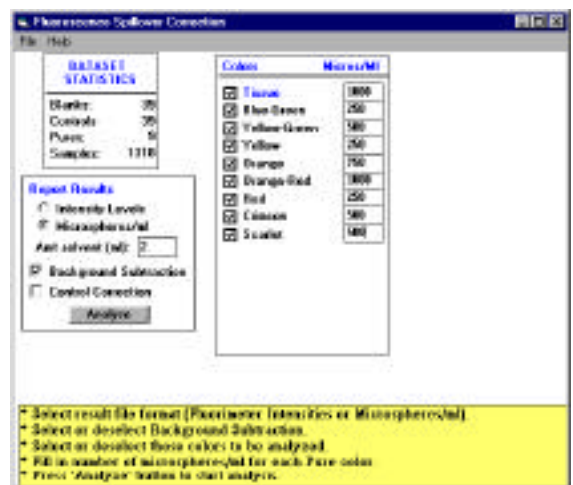


Figure 2: Screen showing the microsphere/ml declarations needed when reporting results in microspheres/ml.

To function properly control solutions should be comprised of all dyes present in the data samples being analyzed. FMSPILL uses simple linear correction to adjust current readings to the value of the initial control reading. One potential pitfall of using control correction is the assumption that intensities of the dyes in the control solution remain constant across time. This may be problematic if sample analysis spans several days and the control sample is not recapped between uses since evaporation of the solvent within the control solution may effectively concentrate the sample and therefore change the control conditions. In this case control correction would not be accounting for electronic drift alone, but rather for electronic drift along with the control evaporation. Therefore the accuracy of the correction is confounded.

Lung tissue, and possibly other tissues, may have significant fluorescence in the blue dye range, which limits the usefulness of blue dye in certain situations. It also presents problems when correcting for spillover, because not only is there significant fluorescence contribution from tissue, but the shape and location of the tissue fluorescence peak is inconsistent between pieces. It is therefore difficult to mathematically correct for tissue fluorescence with any assurance of accuracy. If blue dye is used we recommend reading a tissue window even though mathematical correction using the tissue signal may not be applied. Regardless of use the tissue wavelength readings help to identify samples where tissue spillover may significantly affect the blue dye signal intensity and therefore make those sample results less reliable. If tissue use is requested the program prompts for how to use the data presented. By default, the tissue intensity is merely passed through from the input file to the output file to serve as a marker of possible spillover problems (fig 3). Click the *Use issue in spillover correction option* to include tissue in the spillover matrix. If tissue correction is requested a further dialog box is presented (fig 4). Since each dye reading, including tissue windows, requires a pure sample at the beginning of fluorimetry, the pure sample for a tissue window is a pure solvent sample. The program offers two options on how to deal with those samples: (1) replace the pure sample with an idealized pure sample which is stored in the program, or (2) to use the pure sample provided in the data file.

When all options are set, click the **Analyze** button to proceed. During file processing the computer displays a progress indicator (fig 5). When the analysis is complete the screen returns to the original blank window.

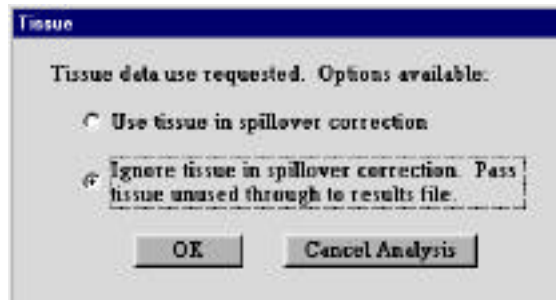


Figure 3: Options available when Tissue data use is requested. By default tissue is not used in spillover

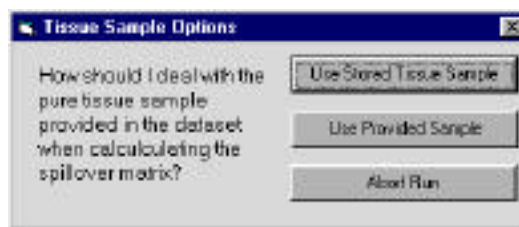


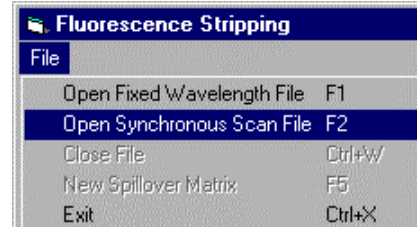
Figure 4: Options available for using Tissue data in spillover correction..



Figure 5: Screen during analysis showing progress bar.

New Spillover Matrix

If the dataset in question does not contain pure samples or if for some reasons you wish to use a different spillover matrix, use the *New Spillover Matrix* option under the *File* menu. You will be prompted to open an .spl file. The format for .spl files is presented in Appendix D.



SYNCHRONOUS SCAN ANALYSIS

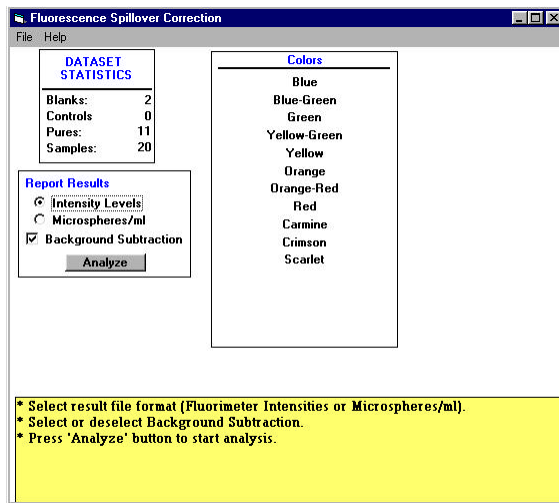


Figure 6: Initial screen for a Synchronous Scan analysis with defaults chosen.

After choosing the *Open Synchronous Scan File* option the program asks for input, blanks and output files. The declaration of files is the same as in fixed wavelength analysis except that the .fix extension is replaced by a .syn extension, the .blk extension is replaced by a .slk extension, and control data is not requested. Adjusting the data through use of control samples is more problematic in a synchronous scan and therefore not provided. After the file declarations are complete, the program performs an initial read through the files. This initial pass determines whether the file(s) are structurally intact. When using Synchronous Scan analysis it is **required** that one pure sample be present in the file for each dye to be analyzed. These samples are used to determine the location of dye peak centers and shape of the peak that are then used to extract intensity levels from the sample data. The program

determines the peak centers and shape of the peaks during this initial read. The cursor spins during this function to assure the user that the program is functioning. The time needed to complete this operation is dependent on both the file size and processor speed of your computer.

At the completion of the initial pass through the data, the results are displayed as shown in figure 6. Unlike the fixed wavelength analysis where the color names are stored within the header of the data file, FMSPILL derives the color names from the location of the peak centers in the pure samples. The program defaults to reporting results in fluorimeter intensity levels. If you desire results reported in microspheres/ml, the number of microspheres/ml that were used to create each of the



Figure 7: Screen display when results are requested in microspheres/ml. The microspheres/ml edit boxes must be filled out prior to continuing the

pure samples must be entered in the appropriate edit boxes (fig 7). The pure samples must be present in the file in the same order from lowest peak wavelength to highest peak wavelength. If there was a blanks file (.slk), the program defaults to using background subtraction. At this point you need to select or deselect the Background Subtraction option. When Background Subtraction is selected, the median intensity of all the blank samples are subtracted point by point from the sample spectrum prior to mathematically correcting the sample for spillover. This removes the contribution of the solvent to the sample intensity. Once all entries are made click the **Analyze** button to proceed.

As the program proceeds each spectrum is displayed and the points used to mathematically correct the sample using the over-determined least squares method are indicated by small circles on each spectrum (fig 8). As the pure samples are analyzed the peak position is used to determine the dye present in the sample. The output file contains the mathematically corrected values for each dye and each record has two additional columns following the color intensities. Those columns, Sum of Squares (SS) and the correlation coefficient (R^2) present two measures of the goodness of fit of the over-determined matrix method. Appendix C presents an example output file from a synchronous scan run.

Since analysis of synchronous scans is slow compared to the fixed wavelength analysis, both Abort and Pause options are available. The Abort (F5) option is present to allow a graceful stoppage of the program prior to completion, whereas the Pause (F11) option allows you to stop and view a scan and then resume the analysis by pressing F11 a second time. F12 toggles the display between drawing each spectrum as it is analyzed and not displaying the spectrum during analysis. The program defaults to displaying the spectrum. The advantage of toggling off the display is a noticeable boost in speed because all the writes to screen are eliminated.

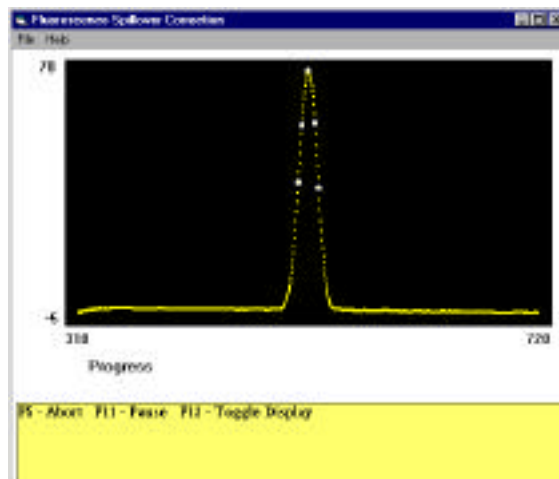


Figure 8: Display during determination of peak centers and shapes. The white circles are the locations that are sampled to determine intensity values in subsequent samples.

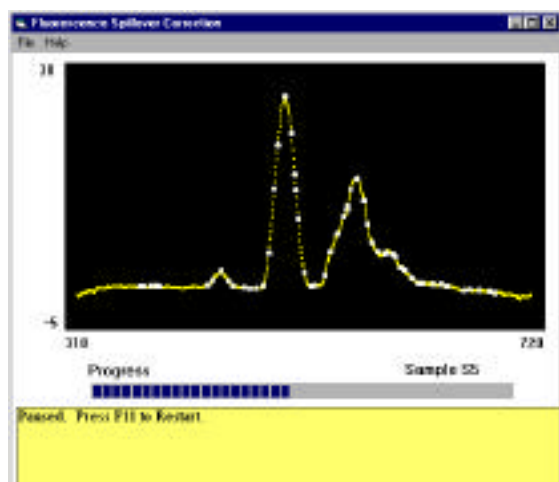


Figure 9:

References

1. Hostetter, G. H., M. S. Santina and P. D'Carpio-Montalvo. Numerical and Computational Methods for Science and Engineering., Englewood Cliffs, New Jersey: Prentice-Hall, 1991
 2. Schimmel, C., D. Frazer, S. R. Huckins and R. W. Glenny. Validation of automated spectrofluorimetry for measurement of regional organ perfusion using fluorescent microspheres. *Comput Methods Programs Biomed.* 62:115-125, 2000.
 3. Schosser, R., K. E. Arfors and K. Messmer. MIC-II - a program for the determination of cardiac output, arterio-venous shunt and regional blood flow using the radioactive microsphere method. *Comput Programs Biomed.* 9:19-38, 1979.
 4. Manual for Using Fluorescent Microspheres to Measure Regional Organ Perfusion, <http://fmrc.pulmcc.washington.edu/fmrc/documents.shtml>.
-

Appendix A: Fixed Wavelength File Format

```
Data~File!tC:\FA\DATA\10SERIE.FIX
Method~name!tC:\FA\METHODS\10SERIES.MTH
Number~of~Wavelengths!t10
Number~of~Pures!t10
Analyst!tcs
Cutoff~Filter!t2
PMT~Voltage!t-1
Sample~Identification!talt~color~dilution~series
Amount~of~solvent!t2
Scan~speed!t~480
Read~time!t~1
Delivery~method!tCuvette
Initial~Date/Time!t09-20-1998!t18:12:28
ex~sl!t4!t4!t4!t4!t4!t4!t4!t4!t4!t4
em~sl!t4!t4!t4!t4!t4!t4!t4!t4!t4!t4
ex~wl!t360!t430!t460!t493!t510!t530!t557!t578!t610!t654
em~wl!t418!t461!t490!t506!t522!t545!t568!t598!t653!t680
B1!t2.898!t1.686!t0.746!t0.226!t0.250!t0.121!t0.000!t0.000!t0.000!t0.000
P1!t46.971!t1.515!t0.739!t0.150!t0.167!t0.060!t0.000!t0.000!t0.000!t0.000
P2!t2.987!t42.625!t1.878!t0.150!t0.416!t0.000!t0.000!t0.000!t0.000!t0.000
P3!t3.002!t2.167!t49.461!t2.107!t0.166!t0.061!t0.000!t0.000!t0.000!t0.000
P4!t2.881!t1.437!t2.382!t78.280!t8.643!t0.060!t0.000!t0.000!t0.000!t0.000
P5!t3.000!t1.611!t0.674!t3.393!t103.732!t1.789!t0.087!t0.000!t0.000!t0.000
P6!t2.892!t1.590!t0.591!t0.225!t1.678!t69.876!t7.241!t0.000!t0.000!t0.000
P7!t3.002!t1.336!t0.596!t0.075!t0.166!t1.702!t69.778!t0.930!t0.000!t0.000
P8!t2.887!t1.604!t0.517!t0.075!t0.083!t0.000!t4.151!t58.829!t0.000!t0.000
P9!t2.884!t1.506!t0.668!t0.075!t0.167!t0.121!t0.000!t0.623!t21.224!t0.000
P10!t3.316!t1.520!t0.668!t0.150!t0.249!t0.000!t0.000!t0.000!t5.468!t70.554
S11!t49.299!t44.418!t59.115!t183.410!t421.504!t83.053!t300.783!t64.866!t21.798!t48.301
S12!t49.860!t44.138!t60.001!t180.968!t422.533!t83.576!t302.654!t65.821!t21.761!t49.492
```

The header is from the first line through the em wl (emission wavelength) declaration. All mandatory lines are in bold. All other lines are not required to be in the header. After the header comes the data. Each record consists of a record label followed by the intensity reading for each wavelength for that record.

Record Label Format:

First Character

B = Blank/solvent only. Must be first record if background subtraction is used.

P = Pure color sample

S = Sample

Successive characters until the first tab character

Sample number

Character Codes Used In Example Above:

!t = Tab character (ASCII 9)

~ = Space

Appendix B: Synchronous Scan File Format

```
Data~File!tC:\FA\DATA\10SERIE.SYN
Method~name!tC:\FA\METHODS\10SERIES.MTH
Number~of~Pures!t10
Analyst!tcs
Cutoff~Filter!t2
PMT~Voltage!t-1
Sample~Identification!talt~color~dilution~series
Amount~of~solvent!t2
Scan~speed!t~480
Scan~lower~limit!t~325
Scan~upper~limit!t~700
Scan~offset!t~15
Scan~data~interval!t~.5
Excitation~slit~width!t~4
Emission~slit~width!t~4
Delivery~method!tCuvette
Initial~Date/Time!t09-20-1998!t18:12:28
B1~.018~.003~.027~.064~.098~.129~.172~.245~.349~.45~.504~.491~.437~...
P1~.006~.02~.05~.099~.168~.246~.321~.382~.426~.465~.509~.556~.598~...
P2~.005~.016~.037~.069~.108~.158~.232~.348~.501~.658~.77~.816~.816~...
P3~.025~.054~.096~.141~.177~.193~.192~.199~.241~.323~.422~.516~.618~...
P4~.039~.076~.124~.183~.25~.314~.356~.355~.311~.253~.232~.293~.447~...
P5~0~0~0~.001~.007~.025~.063~.121~.189~.245~.277~.302~.353~.459~...
P6~.028~.032~.028~.02~.011~.006~.011~.033~.086~.183~.33~.517~.717~...
P7~.036~.063~.088~.098~.088~.064~.039~.026~.033~.063~.118~.197~.295~...
P8~0~0~.002~.009~.027~.063~.123~.217~.344~.489~.615~.684~.681~.622~...
P9~.037~.065~.094~.116~.128~.134~.136~.137~.146~.185~.276~.418~.575~...
P10~.001~.007~.023~.052~.093~.137~.175~.212~.265~.351~.465~.586~.686~...
S1~.006~.02~.05~.099~.168~.246~.321~.382~.426~.465~.509~.556~.598~...
S2~.005~.016~.037~.069~.108~.158~.232~.348~.501~.658~.77~.816~.816~...
S3~.025~.054~.096~.141~.177~.193~.192~.199~.241~.323~.422~.516~...
```

The header is from the first line through the Initial Date/Time declaration. All mandatory lines are in bold. All other lines are not required to be in the header. After the header comes the data. Each record consists of a record label followed by the intensity reading for each increment in the spectra. In the example above the first number is the value at 325 nm and the following number is the value at 325.5 nm, etc. Each record continues until all 751 values for that spectra have been written ((Upper limit - Lower limit)/Scan data interval)+1. The record is ended with a Carriage Return-Line Feed combination (ASCII 13 followed by an ASCII 10 character).

Record Label Format:

First Character

B = Blank/solvent only. Must be first record if background subtraction is used.

P = Pure color sample

S = Sample

Successive characters until the first space character

Sample number

Character Codes Used In Example Above:

!t = Tab character (ASCII 9)

~ = Space

Appendix C: Example Synchronous Scan Output File

All the header information is carried along from the data input file

```

This file has been spillover corrected.
Data File           C:\FA\DATA\10SERIE.SYN
Method name        C:\FA\METHODS\10SERIES.MTH
Number of Pures    8
Analyst            cs
Cutoff Filter      2
PMT Voltage        -1
Sample Identification alt color dilution series
Amount of solvent  2
Scan speed         480
Scan lower limit   325
Scan upper limit   700
Scan offset        15
Scan data interval .5
Excitation slit width 4
Emission slit width 4
Delivery method    AutoSampler
Initial Date/Time  09-20-1998   18:12:28
  
```

The over-determined matrix is listed, with the header row of the matrix listing the wavelengths used in the matrix. The first 5 wavelengths are used to define the first color, 6-10 define the second, etc.

Overdetermined Pure Matrix

Pure/Wavelength	372.00	375.00	379.00	383.00	386.00	434.00	437.00	441.00	445.00...
1	0.38	0.61	1.00	0.91	0.78	-0.06	-0.07	0.02	-0.03...
2	-0.01	0.01	0.02	0.00	0.01	0.75	0.90	1.00	0.88...
3	0.00	0.02	0.02	0.02	0.01	0.00	0.01	0.03	0.03...
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00...
6	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00...
7	0.00	0.00	0.01	0.01	0.00	-0.01	-0.01	0.00	0.00...
8	0.00	0.00	0.01	0.01	0.00	0.00	-0.01	0.00	-0.01...
9	-0.02	0.00	0.01	0.00	0.01	-0.01	-0.01	0.00	0.00...
10	0.00	0.01	0.01	0.02	0.02	0.00	-0.01	0.00	0.00...

Sample/Wl	Blue 379	Blue-Green 441	Green 471.5	Yellow-Green 493	Yellow 509	Orange 537	Orange-Red 553.5	Red 577.5	SS	R^2
P1	8.300	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.00	1.000
P2	0.000	23.121	0.000	0.000	0.000	0.000	0.000	0.000	0.00	1.000
P3	0.000	0.000	34.199	0.000	0.000	0.000	0.000	0.000	0.00	1.000
P4	0.000	0.000	0.000	74.822	0.000	0.000	0.000	0.000	0.00	1.000
P5	0.000	0.000	0.000	0.000	86.156	0.000	0.000	0.000	0.00	1.000
P6	0.000	0.000	0.000	0.000	0.000	97.501	0.000	0.000	0.00	1.000
P7	0.000	0.000	0.000	0.000	0.000	0.000	68.281	0.000	0.00	1.000
P8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	55.108	0.00	1.000
P9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.00	1.000
P10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.00	1.000
S11	1.789	24.526	41.902	151.134	330.058	96.821	281.294	60.036	2368.07	0.996
S12	1.846	24.580	41.965	152.034	333.486	96.560	281.268	59.185	2614.40	0.995
S13	1.734	24.053	42.716	151.442	331.431	96.996	280.056	60.031	2469.49	0.995
S14	2.952	12.552	38.506	77.027	337.285	50.463	282.978	30.474	1074.24	0.998
S15	3.900	13.026	38.568	75.597	337.047	50.347	284.162	30.392	878.66	0.998

Appendix D: Example Spillover Matrix File

The matrix must be composed of the dyes used in the analysis in the order they are present on each record in the dataset.

100.00	0.00	0.36	0.04	0.05	0.07	0.00	0.00	0.00	0.00
0.44	100.00	3.16	0.04	0.66	0.00	0.00	0.00	0.00	0.00
0.40	1.22	100.00	4.04	0.04	0.06	0.00	0.00	0.00	0.00
0.10	0.00	2.31	100.00	10.87	0.04	0.00	0.00	0.00	0.00
0.19	0.04	0.09	3.15	100.00	1.70	0.08	0.00	0.00	0.00
0.12	0.02	0.02	0.13	2.19	100.00	10.37	0.00	0.00	0.00
0.28	0.00	0.02	0.00	0.03	2.40	100.00	1.33	0.00	0.00
0.14	0.05	0.00	0.00	0.00	0.00	7.06	100.00	0.00	0.00
0.37	0.00	0.41	0.00	0.09	0.43	0.00	2.94	100.00	0.00
0.72	0.00	0.12	0.03	0.14	0.00	0.00	0.00	7.75	100.00