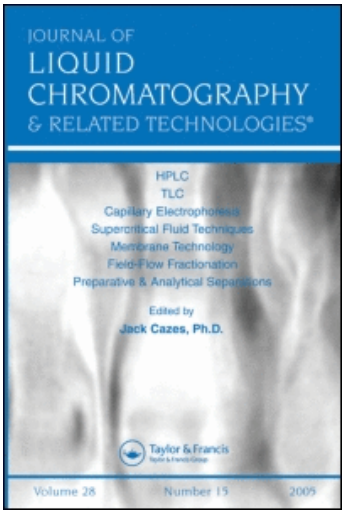

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DIRECT MEASUREMENT OF OCTANOL-WATER PARTITION COEFFICIENTS USING CENTRIFUGAL PARTITION CHROMATOGRAPHY WITH A BACK-FLUSHING TECHNIQUE

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ABSTRACT

Octanol- water partitioning coefficients (K_{oct}) are an accepted parameter in the prediction of biological activity of organic chemicals. Centrifugal Partition Chromatography (CPC), a form of counter current chromatography, is shown to have several distinct advantages in the direct measurement of these partition coefficients. A new CPC technique, back-flushing, is introduced which allows the direct determination of K_{oct} values that previously were thought to be unobtainable by this method. The theoretical and experimental aspects of this technique are presented. K_{oct} of six solutes that ranged from 75 to 3091 were determined and compared with literature values. The direct determination of K_{oct} values greater than 10^4 may be possible. Both accuracy and ease of automation may make this a highly desirable method by which to measure octanol-water partition coefficients in the future.

INTRODUCTION

The n-octanol-water partition coefficient (K_{oct}) of organic compounds has been used as a correlation of their biological activity. This correlation is sometimes referred to as lipophilicity. The Food and Drug Administration and Environmental Protection Agency use the K_{oct} values of organic compounds as a physical parameter to estimate the tendency of these compounds to bioconcentrate in living tissue (1). Researchers have used K_{oct} as a critical physical property for predicting toxicity to aquatic organisms. K_{oct} has been related to absorption of chemicals into sewage sludge and organic particulate matter, and biodegradability of organic chemicals (2). Approximations of K_{oct} can be determined theoretically by using methods developed by Hansch (3) and Free and Wilson (4) if the structure of the compound is known. Quantitative structure reactivity relationships (QSAR) use these methods extensively (5).

The classical shake-flask method is the best experimental way to determine values of K_{oct} (1). A compound is introduced into a bi-phasic system of octanol and water. This is shaken to assure equilibration and the concentration of the compound is measured in both phases. The ratio of the concentration of the compound in the octanol phase to the concentration in the aqueous phase defines K_{oct}. This method has several limitations: i) chemicals with Koct values higher than 1000 ($\log K_{oct} > 3$) cannot be determined with the same precision as chemicals that distribute more evenly between the two phases; ii) mixtures of chemicals cannot be used; iii) for determinations of high K_{oct} values, large amounts of the pure compound are needed.

To extend the range of K_{oct} determination above 1000 by shake-flask, improvements have been proposed. K_{oct} values of up to 10,000 have been

determined using radio labeled compounds. Thermodynamic relationships between K_{oct} and K of other solvent systems have been derived such as:

$$\log K_2 = a \log K_{oct} + b$$

where K_2 is the partition coefficient of the compound in the second solvent system and a and b are constants relating the second system with an octanol-water system. These can be used to compare other parameters such as the hydrogen-bonding ability of a series of compounds (6). Hansch et. al. established equations relating log K_{oct} to different solvent systems (7). Correlation coefficients for these relationships are often poor, however.

Another method that uses a thermodynamic relationship similar to the above relationship is liquid chromatography. The chromatographic log k' of a compound can be related linearly to log K_{oct} (1, 8-10). Relative rapidity, low solute consumption and the ability to determine k' values of compounds in a mixture are the major advantages over the shake-flask method. Unfortunately the correlation between these values and true K_{oct} can be very poor, particularly between different classes of compounds. The same problems are encountered as in the shake-flask method with different solvent systems (1).

Centrifugal Partition Chromatograhy (CPC) has been used to measure partition coefficients (6, 11, 12). Berthod et. al. measured K_{oct} of 17 compounds directly using the basic CPC retention equation

$$V_r = V_m + KV_s$$

where V_r is the retention volume, V_m is the dead volume and V_s is the volume of the stationary phase (12). A K_{oct} value of 167 was the highest

measured directly due to practical limitations such as time and band broadening. Extra-thermodynamic relationships, similar to the relationships already discussed for HPLC, were used to extend the range of K_{oct} values that CPC could determine (6, 12). Testa et. al. concluded that log $K_{oct} > 2$ could not be measured by CPC (13). However in this paper a new technique is introduced. Back-flushing is used to measure directly K_{oct} values between 10^2 to 10^4. The theoretical and experimental aspects of this technique are presented. The K_{oct} values determined compare well with literature values.

EXPERIMENTAL

Equipment

The back-flushing experiments were performed with a centrifugal partition chromatograph, Sanki Model CPC-NMF, from Sanki Laboratories, Inc., Sharon Hill, PA. This apparatus, which can be used with up to 12 cartridges each containing 400 cells, was operated with only 6 cartridges placed in the centrifuge rotor. The design of these cartridges has been thoroughly discussed previously (14). This rotor is housed in a constant temperature box, which was maintained at 20°C.

The Sanki command module (Model CPC-FCU-V) was not used in this application as had been done in previous CPC applications. The back-flushing technique described here required that the "column" be isolated from the rest of the system during mobile phase conversion. This requires a three valve manifold. Figure 1 shows a schematic of this manifold. A 6-port Rheodyne Model 7010 valve with a 20 µl sample loop was used to introduce the sample. Two Rheodyne Model 7000 valves were used as mode selection and by-pass valves. Although these are 6 port valves, they were operated as 4 port valves by simply linking two ports on each valve. The

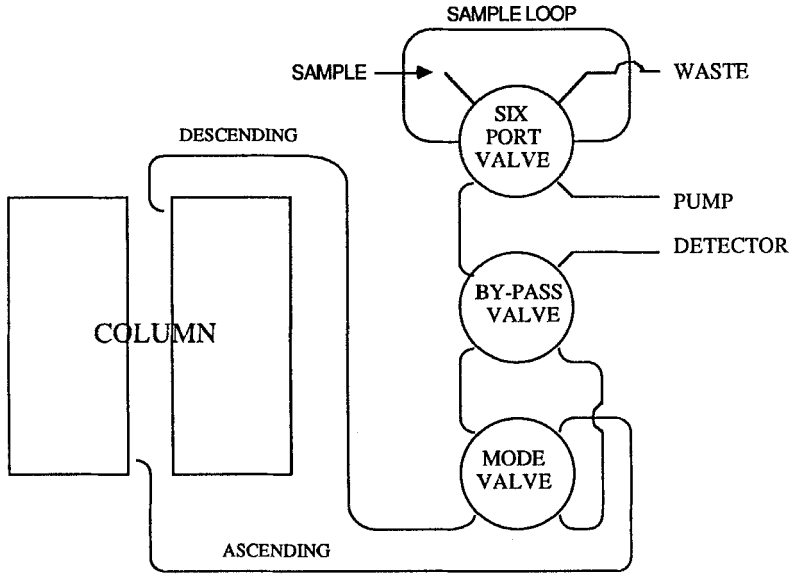

Figure 1. Schematic of 3 valve manifold used in the back-flushing experiments. The by-pass valve is used to isolate the system and the mode valve is used to select the ascending or descending mode.

total internal volume of the system, consisting of the 3 valve manifold and 6 cartridges, is 120 ml.

A LC pump (Shimadzu LC-5A) was used. The detector was a Shimadzu, Model SPD-6A, UV-vis detector equipped with a standard analytical flow cell. The response factor of the detector was the slow setting. A Linear Inc., Model 1200 recorder was used.

Materials

HPLC grade 1-octanol was obtained from Aldrich Chemical Co., Inc., and was used without further purification. The water was distilled and passed

through a Barnstead D8922 cartridge to trap organics and filtered through a 0.45μ Alltech nylon 66 membrane to remove particulates. All solvents were mutually saturated before being used. The pH of the aqueous phase was 2.0 for the solute benzoic acid in order to assure the acid remained protonated. Benzoic acid, ethyl benzoate, naphthalene, 1-naphthol, and toluene were purchased from Aldrich and were used without purification. o-Chlorophenol was obtained from Dow Chemical (Midland, MI.).

THEORY

The following statistical theory is sufficient for describing the locations of peak maxima. More detailed analysis of peak shape requires a differential theory which will be presented in a future publication. The "column" is assumed to consist of P* cells, each containing a volume of stationary phase, V_S, and a volume of mobile phase, V_m. A solute is injected into the mobile phase, and allowed to flow in this "forward" mode at some flow rate, v_f', for a period of time, t_f. The forward volume of flow, V_f, is then :

$$V_f = v_f' t_f \qquad [1]$$

In this forward flow volume, the number of exchanges, E_f, that take place are given by:

$$E_f = V_f / V_m \, . \qquad [2]$$

The partition coefficient, K, is defined as the concentration of the solute in the octanol phase, C_o, divided by the concentration in the aqueous phase, C_{aq}, can be written in terms of the fraction of the solute in the stationary phase, f_s, and the mobile phase $(1 - f_s)$.

$$K = C_o / C_{aq} = [\, f_s V_m] / [\, (1 - f_s) V_s] \qquad [3]$$

Rearrangement yields:

$$f_s = K(V_s / V_m) / [1 + K(V_s / V_m)] \,. \qquad [4]$$

After E_f exchanges in the forward mode, the amount of solute (n) (relative to the total amount of solute, n^o) in any cell (P) is given by:

$$[\, n/n_o]\, \{P, E_f\} = [\, f_s^{E_f - P + 1} (1 - f_s)^{P - 1} E_f! \,] / \qquad [5]$$
$$[(E_f - P + 1)!\, (P - 1)!] \,.$$

The flow of the mobile phase is stopped before any solute reaches the final cell. The role of the mobile and stationary phases is reversed and the new mobile phase is pumped in the opposite direction, back-flushing the column. The original subscripts will be retained, except the subscripts s and m now refer to the mobile and stationary phases, respectively. The number of exchanges in the reverse flow, E_r, is a function of the volume of flow in the reverse direction, V_r, in a similar manner as the forward exchanges:

$$E_r = v_r' t_r / V_s = V_r / V_s \,. \qquad [6]$$

Upon reversing the flow, the solute eluting to the detector is the material of the first cell, P = 1. The amount of solute, relative to the total amount of solute at the detector is:

$$[\, n/n_o]_{detector} = f_s [\, n/n_o]\, \{P=1, E_f, E_r\} . \qquad [7]$$

In the appendix, this is shown to be:

$$[n/n_o]_{detector} = f_s E_f + 1(1 - f_s) E_f (E_f + E_r)! / (E_f! E_r!) \quad [8]$$

Using Stirling's approximation and locating the band maxima with respect to the number of exchanges in the reverse direction:

$$f_s / (1 - f_s) = (E_f / E_r)_{max} \quad [9]$$

Substituting from equations 2 and 6 :

$$K = f_s V_m / (1 - f_s) V_s = V_f / V_r \quad [10]$$

Thus it is shown that the partition coefficient is simply the ratio of the forward and reverse volumes. The derivations of these equations involve three assumptions:

1. The volume of the injected sample is very small compared to the total forward flow volume.

2. Volumes of stationary and mobile phases in cells containing significant quantities of solute are not changed by the reversal of flow.

3. No solute leaves the column during the forward mode. More specifically, no significant amount of solute should reach a cell within some critical volume of stationary phase from the end of the column. This critical volume represents the volume of mobile phase in the connecting piping between the last cell and the inlet for reversed flow. An equivalent volume of stationary phase will be flushed out the cell starting from the end of the column as the direction of flow is reversed.

PROCEDURE

Forward

The cartridges were loaded with the initial stationary phase, octanol, in a manner similar to the previously described "underload" method (12). First, the cartridges are filled entirely with octanol in the descending mode without rotation. Next, the mode is changed to the ascending mode and rotation is set at 200 RPM. Water is then introduced by pumping in the direction opposite to that recommended by the manufacturer, to displace the octanol. The displaced octanol is collected in a graduated cylinder. The percentage of octanol in the system is controlled by the amount of octanol displaced. Since the phase ratio does not effect the final result, the choice of the phase ratio is based entirely on the operating pressures and flooding flow rates. When the desired amount of octanol has been displaced, the mode is switched to descending and rotation is increased to operating speed. This causes a "flooding" (15) condition in the head of the "column", but this flooding loads the tail of the column which has a low percentage of octanol. This flooding can be controlled by the flow rate and rotation speed. A forward flow rate of 4 ml/min or 3ml/min was used. The accuracy of both the forward and the reverse flow rates were measured. The forward flow rates were determined to be 3.90 $\pm$ 0.07 ml/min and 2.97 $\pm$ 0.02 ml/min for 4 and 3 ml/min respectfully. These were determined by measuring the amount of time needed to fill a grade A volumetric flask. A rotation speed of 900 RPM was used. The "column" was allowed to equilibrate before introduction of the solute. 5µl samples were introduced with a micro syringe into a 20 µl sample loop. The size of the sample must be kept small (i.e. < 5µl). If the sample size is increased beyond this size, a "flooding" condition in the first few cells may exist. This would allow the solute to move down the "column" farther

than the theory would predict, thus resulting in a Koct value larger than is real.

Reversal

The reversal of the direction of flow requires the changing of the mobile phase from water to octanol. This is initiated by first isolating the "column" while maintaining rotation. Isolating the "column" is accomplished with the by-pass valve of the manifold. Isolating the column allows two things: first, the column is now static (no movement of solvent or solute is taking place between each cell); secondly, the pump and all tubing can be flushed free of water to reduce signal noise when flow is resumed through the column. A small volume of mobile phase is needed to flush the remaining water from the tubing between the column head and the valve manifold. This volume was calculated to be less than 0.15 ml in this system. Once the pump and tubing have been primed with octanol, then the flow may begin through the column in the ascending mode. The flow rate of 0.3ml/min was used for the reverse flow rate. This flow rate was determined, as in the forward procedure above, to be 0.288 ± 0.008 ml/min.

RESULTS AND DISCUSSION

The aim, as stated before, is the direct measurement of octanol-water partition coefficients. The range of directly measured octanol-water partition coefficients by CPC has been extended to 3091 in this work and it is estimated to be possible to extend this to 10,000 and higher by the back-flushing method. A limit of 24 hours for t_f was set on all experiments. Longer periods of t_f could be used if desired. The flow rates of the mobile phases were set by the pressure limit of the instrument and the flooding flow

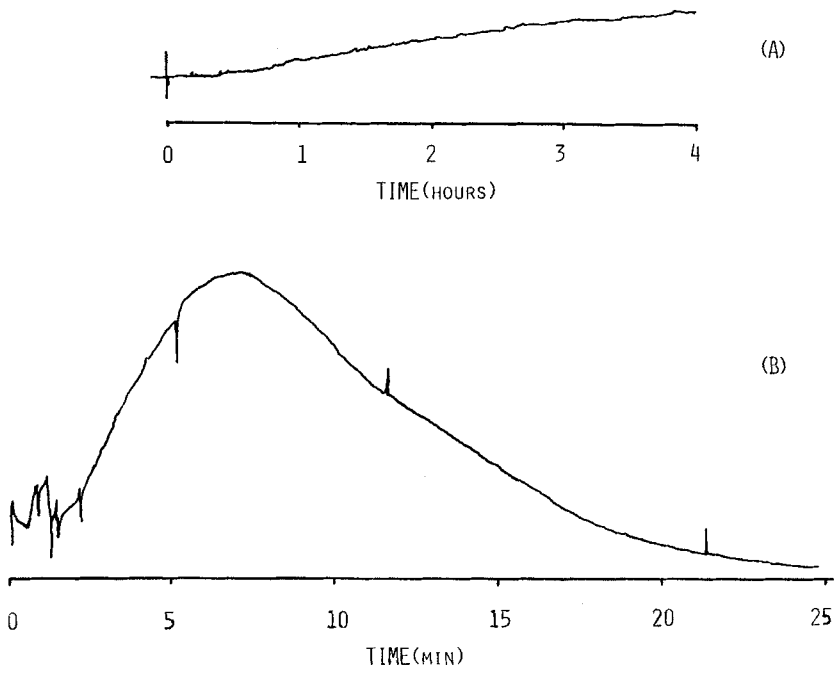

Figure 2. Chromatogram from a back-flushing experiment of toluene. (A) Forward mode; flow rate is 4.0 ml/min.. (B) Reverse mode; flow rate is 0.3 ml/min..

rates. Flooding in the reverse mode causes the formation of an emulsion in the detector from the head of the column. Noise from an octanol water emulsion obscures the signal of the solute at the detector. The flooding flow rate could be increased with increased rotation speed but the pressure increases proportionally with the square of rotation speed (11). Therefore, a compromise must be found for the flow and rotation rates. The pressure also is dependent on the height of the stationary phase, which is a function of the percentage of octanol in the system. The maximum flow rate of water is

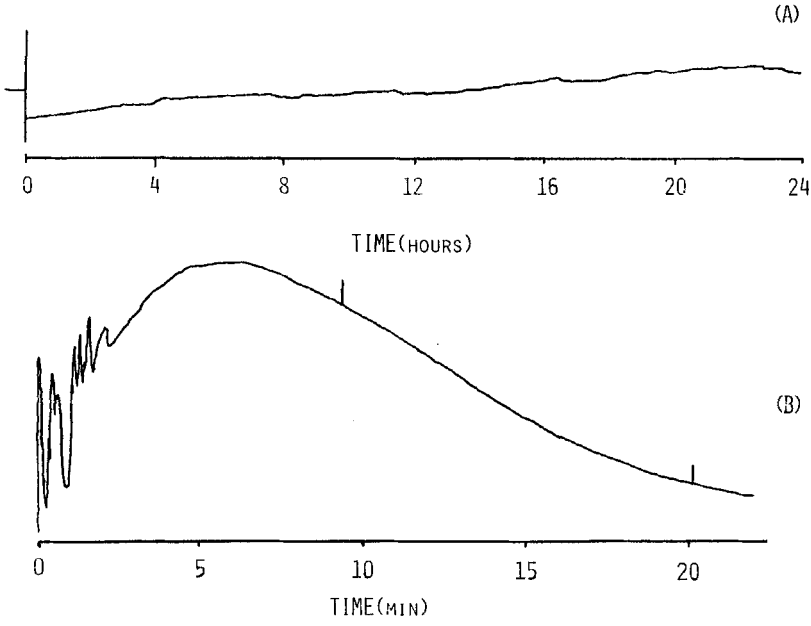

Figure 3. Chromatogram from a back-flushing experiment of naphthalene. (A) Forward mode; flow rate is 4.0 ml/min.. (B) Reverse mode; flow rate is 0.3 ml/min..

much greater than the maximum flow rate of octanol due to viscosity. Empirically, it was found that greater than 50% octanol in the column increased pressure beyond the limit of the instrument while less than 35% in the system increased noise at the detector beyond the ability to detect band maxima, upon reversal of the flow. The range of 35 -50% octanol in the system was used in all experiments. This range can and will change with different systems. Upon reversal of flow, the volume of water from the head of the column to the valve manifold is eluted to the detector. This creates substantial noise at the detector. Determination of high K's require the use of

TABLE I. Typical Forward and Reverse Volume and Average K_{oct}

Solute	V_f	V_R	K_{oct}[a]	log K_{oct}[a]	lit log K[b]
Benzoic Acid[c]	178.0	2.36	75.4 ± 3.7	1.88	1.87
o-Chlorophenol	487.3	2.71	179.8 ± 4.3	2.25	2.15 - 2.19
Toluene	1072	2.59	414.8 ± 49	2.62	2.11 - 2.80
Ethyl Benzoate	1169	1.96	592.4 ± 60	2.77	NA
1-Naphthol	2924	2.16	1355 ± 148	3.13	2.84 - 2.98
Naphthalene	4795	1.58	3091 ± 91	3.49	3.01 - 3.45

[a] K_{oct} and log K_{oct} are calculated averages of 3 or 4 separate experiments. The V_f/V_R shown may not give K_{oct} and log K_{oct} but are typical values of V_f and V_R for that solute.
[b] See reference 7.
[c] The pH of the aqueous phase was 2.0.

large amounts of forward mobile phase (water), thus making the recycling of solvents advantageous (16). The recycling of solvents also reduces "column" bleeding.

The octanol-water partition coefficients of six solutes; benzoic acid, ethyl benzoate, 1-naphthol, naphthalene, o-chlorophenol, and toluene were determined directly by back-flushing. The K_{oct} of these solutes have a range that extends up to 3091. Figures 2 and 3 show chromatographs of typical experiments for toluene and naphthalene, respectfully. Approximate K's were determined for solutes prior to the experiment to determine how long to pump in the forward direction (7). After an initial experiment the V_f was

adjusted to shift band maximum of the solute in the reverse mode, V_r. Table I lists typical values of V_f and V_r. The calculated K's are the average of 3 or 4 separate experiments. Each experiment may have a different V_f. V_f's were adjusted so that the V_r could be determined as accurately as possible. Standard deviations for K_{OCT} of less than 11% were obtained.

The maximum K that could be determined accurately depends on the ability to measure the volumes V_f and V_r. Since the measurement of larger volumes of flow is more accurate, by percentage, than a small volume of flow, the determination of V_r is the limiting parameter. If a minimum limit of 1.2ml (4 min at 0.3 ml/min) is placed on $V_{r_{max}}$ for a V_f of 11.5 liters (48 hours at 4 ml/min) then a K of approximately 10,000 could be determined. V_r's shorter than this cause the band maximum to be mixed with the noise of the reversal and accurate measurements of $V_{r_{max}}$ is hindered. V_f can be increased with one restriction. Exchanges forward, E_f, as defined in equation 2, increases with V_f. Equation [A1] in the appendix shows that the solute band will broaden with increases in E_f and E_r. E_r is small when V_r is small. E_f is much greater than P when V_f is large. Therefore $[n/n_O]$ decreases with increasing V_f. This decreases the ability to detect the band maximum. Increasing the amount of the solute, n_O, allows a slight improvement in $V_{r_{max}}$ detection.

ACKNOWLEDGEMENTS

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APPENDIX

Mass balance requires of the reversed flow that :

$$[\, n/n^{o}] \{P, E_f, E_r\} = [\, n_s/n^{o}] \{P+1, E_f, E_r-1\} + [\, n_m/n^{o}] \{P, E_f, E_r-1\}$$

$$[\, n/n^o] \{P, E_f, E_r=1\} = [\, n_s/n^o] \{P+1, E_f, E_r=0\} + [\, n_m/n^o] \{P, E_f, E_r=0\}$$

$$[\, n/n^o] \{P, E_f, E_r=1\} = f_s[\, f_s^{E_f - P} (1-f_s)^P E_f! / (E_f - P)!\, P!\,] +$$

$$(1-f_s)\,[\, f_s^{E_f - P+1} (1-f_s)^{P-1} E_f! / (E_f - P + 1)!(P-1)!]$$

This can be manipulated to the form:

$$[\, n/n^o] \{P, E_f, E_r=1\} = f_s^{E_f - P+1} (1-f_s)^P (E_f + 1)! \;/\; (E_f - P + 1)!\, P!$$

Again, mass balance requires that:

$$[\, n/n^o] \{P, E_f, E_r=2\} = [\, n_s/n^o] \{P+1, E_f, E_r=1\} + [\, n_m/n_o] \{P, E_f, E_r=1\}$$

and similar treatment of this yields:

$$[\, n/n^o] \{P, E_f, E_r=2\} = [\, f_s^{E_f - P + 1} (1 - f_s)^{P+1} (E_f + 2)!\,] / (E_f - P + 1)!(P+1)!$$

Generalization of this sequence gives:

$$[\, n/n^o] \{P, E_f, E_r\} = [\, f_s^{E_f - P + 1} (1 - f_s)^{P + E_f - 1} (E_f + E_r)!\,] / \quad [A1]$$
$$(E_f - P + 1)!\,(P + E_r - 1)!$$

From equation 7:

$$[\, n/n^o]_{detector} = f_s[\, n/n^o] \{P=1, E_f, E_r\}\,.$$

Substitution of P =1 in equation A1 gives :

$$[\, n/n^o] \{P, E_f, E_r\} = f_s^{E_f} (1 - f_s)^{E_f} (E_f + E_r)! / E_f!\, E_r!$$

and

$$[\, n/n^o]_{detector} = f_s^{E_f + 1} (1 - f_s)^{E_f} (E_f + E_r)! / E_f!\, E_r!$$