



(86) Date de dépôt PCT/PCT Filing Date: 2005/06/21  
(87) Date publication PCT/PCT Publication Date: 2006/01/26  
(45) Date de délivrance/Issue Date: 2011/08/23  
(85) Entrée phase nationale/National Entry: 2006/12/01  
(86) N° demande PCT/PCT Application No.: US 2005/021743  
(87) N° publication PCT/PCT Publication No.: 2006/009989  
(30) Priorité/Priority: 2004/06/22 (US60/581,824)

(51) Cl.Int./Int.Cl. *C12N 9/74* (2006.01),  
*C12N 9/96* (2006.01)  
(72) Inventeurs/Inventors:  
JIANG, SHAN, US;  
SENDEROFF, RICHARD I., US;  
MEYER, JEFFREY D., US  
(73) Propriétaire/Owner:  
ZYMOGENETICS, INC., US  
(74) Agent: SMART & BIGGAR

(54) Titre : COMPOSITIONS DE THROMBINE  
(54) Title: THROMBIN COMPOSITIONS

(57) **Abrégé/Abstract:**

Compositions comprising thrombin and methods of preparing them are disclosed. The composition may be in the form of an aqueous solution at pH 5.7 - 7.4 consisting essentially of 0.1 mg/mL to 5.0 mg/mL thrombin, 2% to 4% (w/v) sucrose, 3.5% to 5% (w/v) mannitol, 50 mM to 300 mM NaCl, 0-5mM CaCl<sub>2</sub>, 0.03% to 1% (w/v) of a surfactant or high-molecular-weight polyethylene glycol, and a physiologically acceptable buffer, or may be in lyophilized form.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
26 January 2006 (26.01.2006)

PCT

(10) International Publication Number  
**WO 2006/009989 A1**(51) International Patent Classification<sup>7</sup>: C12N 9/74, 9/96(21) International Application Number:  
PCT/US2005/021743

(22) International Filing Date: 21 June 2005 (21.06.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/581,824 22 June 2004 (22.06.2004) US(71) Applicant (for all designated States except US): ZYMO-  
GENETICS, INC. [US/US]; 1201 Eastlake Avenue East,  
Seattle, WA 98102 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): JIANG, Shan  
[CN/US]; 3502 211th Place, SE, Sammamish, WA 98075  
(US). SENDEROFF, Richard, I. [US/US]; 18823 81st  
Avenue West, Edmonds, WA 98026 (US). MEYER,  
Jeffrey, D. [US/US]; 5450 NE 200th Place, Lake Forest  
Park, WA 98155 (US).(74) Agents: PARKER, Gary, E. et al.; 1201 Eastlake Avenue  
East, Seattle, WA 98102 (US).(81) Designated States (unless otherwise indicated, for every  
kind of national protection available): AE, AG, AL, AM,  
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,  
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,  
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,  
KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA,  
MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ,  
OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL,  
SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC,  
VN, YU, ZA, ZM, ZW.(84) Designated States (unless otherwise indicated, for every  
kind of regional protection available): ARIPO (BW, GH,  
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,  
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,  
FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO,  
SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN,  
GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

WO 2006/009989 A1

(54) Title: THROMBIN COMPOSITIONS

(57) Abstract: Compositions comprising thrombin and methods of preparing them are disclosed. The composition may be in the form of an aqueous solution at pH 5.7 - 7.4 consisting essentially of 0.1 mg/mL to 5.0 mg/mL thrombin, 2% to 4% (w/v) sucrose, 3.5% to 5% (w/v) mannitol, 50 mM to 300 mM NaCl, 0-5mM CaCl<sub>2</sub>, 0.03% to 1% (w/v) of a surfactant or high-molecular-weight polyethylene glycol, and a physiologically acceptable buffer, or may be in lyophilized form.

Description

## THROMBIN COMPOSITIONS

## BACKGROUND OF THE INVENTION

Formulation of pharmaceutical proteins presents significant challenges. Proteins possess multiple functional groups in addition to three-dimensional structure; degradation therefore proceeds via both chemical (modifications involving bond formation or cleavage) and physical (denaturation, aggregation, adsorption, precipitation) pathways. Since each protein embodies a unique combination of amino acid sequence, isoelectric point, and other determinants, its response to changes in solution conditions is unpredictable, and must be determined on a case-by-case basis. Attempts to prevent one form of degradation often increase the rate of another.

Degradation of proteins can be greatly reduced or avoided by lyophilization. However, lyophilization is time-consuming and costly, and can cause protein denaturation and aggregation if appropriate excipients are not included. As with solution formulation, stabilization of lyophilized proteins must be dealt with on an individual basis.

In the case of thrombin, sodium chloride can be used to maintain stability during purification and storage by reducing aggregation and precipitation. However, sodium chloride is a problematic excipient in lyophilized formulations because it lowers glass transition temperature, thereby necessitating low primary temperatures and long cycle times. In addition, thrombin must be protected from unfolding and aggregation within the lyophilized composition.

The ideal formulation will combine stability during lyophilization, as well as long-term storage and shipment with ease of handling and use.

## DESCRIPTION OF THE INVENTION

Within one aspect of the invention there is provided a composition consisting essentially of 0.1 mg/mL to 5.0 mg/mL thrombin, 2% to 4% (w/v) sucrose, 3.5% to 5% (w/v) mannitol, 50 mM to 300 mM NaCl, 0-5mM CaCl<sub>2</sub>, 0.03% to 1% (w/v) of a surfactant or high-molecular-weight polyethylene glycol, and a physiologically acceptable buffer, in aqueous solution at pH 5.7 - 7.4, wherein concentration of the buffer is selected to provide approximately physiological pH upon application of the composition in a surgical setting and wherein the ratio of

mannitol:sucrose is greater than 1:1 but not greater than 2.5:1 (w/w). Within one embodiment of the invention the ratio of mannitol to sucrose is 1.33:1 (w/w). Within other embodiments of the invention the molar ratio of sucrose:thrombin is at least 700:1 or at least 2000:1. Within further embodiments of the invention the thrombin is human thrombin, including, for example, recombinant human thrombin. Within still other embodiments of the invention, the thrombin concentration is 0.5 - 3.0 mg/ml or the thrombin concentration is 1 mg/ml. Within other embodiments of the invention, the sucrose concentration is 3% (w/v) and/or the mannitol concentration is 4% (w/v). Within a further embodiment the surfactant or high-molecular-weight polyethylene glycol is PEG 3350. Within a related embodiment the surfactant or high-molecular-weight polyethylene glycol is PEG 3350 at a concentration of 0.1% (w/v). Within additional embodiments, the pH of the composition is 6.0. Within further embodiments the buffer is selected from the group consisting of histidine, citrate, phosphate, Tris, and succinate buffers. Within related embodiments the buffer is histidine buffer, such as 2.0-10 mM histidine buffer or 5 mM histidine buffer. One such composition of the present invention consists essentially of 0.1 mg/mL to 5.0 mg/mL thrombin, 2% to 4% (w/v) sucrose, 3.5% to 5% (w/v) mannitol, 100 mM to 200 mM NaCl, 1 mM to 5 mM CaCl<sub>2</sub>, 0.03% to 1% (w/v) PEG3350, and 2 mM to 10 mM histidine in aqueous solution at pH 5.5 - 6.5, wherein the ratio of mannitol:sucrose is greater than 1:1 but not greater than 2.5:1 (w/w). A second such composition of the invention consists essentially of 1 mg/ml thrombin, 3% (w/v) sucrose, 4% (w/v) mannitol, 4 mM CaCl<sub>2</sub>, 0.1% (w/v) PEG3350, 150 mM NaCl, and 5 mM histidine in aqueous solution at pH 6.0.

Within a second aspect of the invention there is provided a method of preparing a lyophilized thrombin composition comprising (a) providing an aqueous, thrombin-containing composition as disclosed above and (b) lyophilizing the aqueous composition to form a lyophilized thrombin composition.

Within a third aspect of the invention there is provided a lyophilized thrombin composition prepared by the method disclosed above. Within one embodiment of the invention the lyophilized composition is contained in a sealed container having a label affixed to an exterior surface thereof. Within an additional embodiment of the invention the composition in the sealed container contains from 2,500 to 20,000 NIH units of thrombin. Within a related embodiment of the invention the composition is provided in the form of a kit comprising a sealed container as disclosed above and a second sealed container containing a diluent such as, for example, normal saline.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention and the attached drawings. In the drawings:

Fig. 1 illustrates the analysis of pre- and post-lyophilization samples of certain thrombin formulations by Right Angle Light Scattering (RALS) .

Fig. 2 illustrates the analysis of certain thrombin formulations by RALS following storage for thirteen weeks.

Fig. 3 illustrates the analysis of certain thrombin formulations by RALS following storage for six months.

Fig. 4 illustrates the analysis of thrombin secondary structure by Fourier transform infrared spectroscopy using four formulations of recombinant human thrombin (labeled A, B, C, and D).

Fig. 5 illustrates the analysis of thrombin secondary structure by Fourier transform infrared spectroscopy using five formulations of recombinant human thrombin (labeled T, A, B, C, and D).

Numerical ranges (e.g., "from X to Y") include the endpoints unless otherwise specified.

The present invention provides stabilized thrombin compositions, including lyophilized compositions. As used herein, "thrombin" denotes the activated enzyme, also known as  $\alpha$ -thrombin, which results from the proteolytic cleavage of prothrombin (factor II). As disclosed below, thrombin can be prepared by a variety of methods known in the art, and the term "thrombin" is not intended to imply a particular method of production. Human thrombin is a 295 amino acid protein composed of two polypeptide chains joined by a disulfide bond. Both human and non-human thrombins can be used within the present invention. Thrombin is used medically as a hemostatic agent and as a component of tissue adhesives.

Human and non-human (e.g., bovine) thrombins are prepared according to methods known in the art. Purification of thrombin from plasma is disclosed by, for example, Bui-Khac et al., U.S. Patent No. 5,981,254. Purification of thrombin from plasma fractions, such as Cohn fraction III, is disclosed by Fenton et al., *J. Biol. Chem.* 252:3587-3598, 1977. Recombinant thrombin can be prepared from a prethrombin precursor by activation with a snake venom activator as disclosed in U.S. Patent No. 5,476,777. Suitable venom activators include ecarin and prothrombin activator from *Oxyuranus scutellatus*.

Within the present invention, thrombin is formulated in a weakly buffered, aqueous solution containing sucrose, mannitol, sodium chloride, a surfactant or high molecular weight polyethylene glycol (HMW-PEG), and, optionally, calcium

chloride. Concentration ranges of these components are shown in Table 1. Concentrations expressed as percent are on a weight-to-volume basis.

Table 1

thrombin	0.01-5.0 mg/mL
sucrose	2% - 4%
mannitol	3.5% - 5%
NaCl	50-300 mM
surfactant or HMW-PEG	0.03%-1%
CaCl <sub>2</sub>	0-5 mM
pH	5.7-7.4

The concentration of thrombin within the formulation of the present invention can be varied depending on the intended use, including the desired concentration of the reconstituted product. Thrombin is included in the formulation at a concentration of 0.01-5.0 mg/mL, more commonly 0.1-5.0 mg/mL, and typically 0.5-3.0 mg/mL. Within certain embodiments of the invention the concentration of thrombin is 1.0 mg/mL.

The inventors have found that the ratio of mannitol to sucrose significantly affects protein unfolding during lyophilization. Mannitol is included as a bulking agent, to improve lyophilization cycle efficiency and lyophilized product appearance. Sucrose is included as a stabilizer (lyoprotectant), reducing thrombin unfolding during lyophilization and upon storage. Formulations having a mannitol:sucrose ratio of 5:1 (w/w) or greater were unacceptable; those having a ratio of 2.5:1 or less appeared stable as analyzed by Fourier transform infrared spectroscopy (FTIR). Ratios of 1:1 or less showed aggregation/precipitation in solution (upon reconstitution) as measured by right angle light scattering (RALS) and did not form an acceptable cake upon lyophilization. Thus, within the present invention the ratio of mannitol:sucrose will be greater than 1:1 but not greater than 2.5:1.

NaCl is included in the formulation to reduce aggregation and/or precipitation. As noted above, NaCl can be problematic in the lyophilization process. Within the formulation of the present invention, NaCl is included at a concentration of 50 mM to 300 mM. Within certain embodiments of the invention the concentration of NaCl is 75 mM to 250 mM, 100 mM to 200 mM, or 150 mM.

The thrombin formulation of the present invention is buffered to provide for stability during formulation and to optimize activity upon reconstitution. While not wishing to be bound by theory, it is believed that autolytic degradation of thrombin

increases as pH increases above 6.0, and the precipitation rate increases as pH decreases below 6.0. It is therefore desirable to prepare thrombin at slightly acidic to approximately neutral pH to limit loss of activity due to autolysis (formation of inactive autolytic degradation products including  $\beta$ - and  $\gamma$ -thrombin). However, to provide optimal biological activity upon use, approximately physiological pH is desired. The present invention addresses these conflicting needs through the use of a weak buffer that is effective at slightly acidic to approximately neutral pH (i.e., pH 5.7-7.4), but will allow the pH to reach approximately physiological pH (i.e., pH 6.8 - 7.5, preferably pH 7.0 - 7.5, more preferably pH 7.35 - 7.45) when the thrombin is applied to bleeding tissue in a surgical setting. Within the present invention the thrombin solution is buffered at pH 5.7-7.4, commonly 6.0-6.5, and, within certain embodiments, 6.0. Suitable buffers include, in addition to histidine, other physiologically acceptable buffers that are effective within the noted pH range and will allow the solution to reach approximately physiological pH upon application of the composition in a surgical setting. Examples of such buffers include phosphate, citrate, Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol), and succinate. Within certain embodiments of the invention, histidine buffer is included at 1-20 mM, 2-10 mM, or 5 mM.

Suitable concentrations of other buffers within the present invention can be readily determined by one of ordinary skill in the art. Formulation buffers can be tested by adding blood and measuring the pH of the resulting solution. In an exemplary assay, aliquots of rabbit blood are added stepwise to various buffers, and pH is measured after each addition. When histidine buffers are tested in such an assay, 3.2 mM, 5 mM, 12.8 mM, and 20 mM histidine are neutralized by the addition of not more than 1.0 volume of blood. In contrast, 160 mM succinate buffer and 90 mM phosphate/12.8 mM histidine buffer did not produce a mixture with a pH above 7.0 even after addition of 2-3 volumes of blood.

Within the present invention, a surfactant or high-molecular-weight polyethylene glycol (PEG) is included to prevent adsorptive losses and shear-induced thrombin aggregation/precipitation in solutions prior to lyophilization or after reconstitution of the lyophilized product. Suitable surfactants useful in this regard include polyethylene oxides, sorbitan esters, polyoxyethylene alkyl ethers, glycerides of fatty acids (e.g., glyceryl monooleate and glyceryl monostearate), polyoxyethylene sorbitan fatty acid esters (e.g., polysorbate 20 (polyoxyethylene sorbitan monolaurate) and polysorbate 80 (polyoxyethylene sorbitan monooleate)), and the like. High-molecular-weight polyethylene glycols include those having molecular weights from 400 to 8000, such as PEG 400, PEG 1000, PEG 3350, PEG 5000, and PEG 8000. An exemplary high-molecular-weight polyethylene glycol is PEG 3350. The preferred

concentration of PEG 3350 is 0.1-1%. Although 0.03% PEG 3350 effectively inhibits adsorptive losses and shear-induced aggregation/precipitation, somewhat higher concentrations will commonly be used in the initial formulation to account for dilution following reconstitution. For example, a typical unit dose of thrombin can be prepared by filling a vial with 1.6 mL of a 1.0 mg/mL (3200 U/mg) solution (about 5000 NIH U/vial) and lyophilizing. The lyophilized product, when reconstituted with 5 mL of normal saline, provides a thrombin concentration of about 1000 NIH U/mL (0.32 mg/mL). If the concentration of PEG 3350 in the initial formulation is 0.1%, the concentration in the reconstituted product will be 0.03%.

Within certain embodiments of the invention,  $\text{CaCl}_2$  is included in the formulation to promote hemostasis, as it is required to activate several blood clotting factors (e.g., Factor IX, Factor X). When included, the concentration of  $\text{CaCl}_2$  in the formulation is up to 5 mM, generally 1-5 mM, commonly 4 mM. Inclusion of calcium chloride may also improve the physical stability of thrombin.

For long-term storage, the aqueous solution is aliquoted into sterile vials, ampoules, or other containers and lyophilized according to procedures known in the art. The lyophilized product appears as a powder or cake, a cake being the preferred form. The containers are then sealed. It is preferred to use a seal that permits later injection of diluent through the seal and into the container. The container is labeled according to standard practice in the pharmaceutical field.

In one embodiment of the invention, the container is provided in a kit with a second container containing a diluent. Suitable diluents include normal saline for injection and water for injection. The kit may further comprise an application device, such as a sprayer, syringe, or the like.

For use, the lyophilized thrombin composition is reconstituted with a suitable diluent to the desired concentration, generally from about 100 NIH U/ml to about 5,000 NIH U/ml, typically about 1,000 NIH U/ml, although the actual concentration will be determined by the physician according to the needs of the individual patient. The thrombin can be applied to bleeding tissue to achieve hemostasis, often in combination with an absorbable gelatin sponge. The thrombin can also be used as a component of a tissue adhesive or fibrin glue. These and other uses of thrombin are known in the art.

The invention is further illustrated by the following non-limiting examples.



## EXAMPLES

Example 1

Recombinant human thrombin (rhThrombin) was formulated at 1 mg/ml in 5 mM histidine, 150 mM NaCl, 4 mM CaCl<sub>2</sub>, 0.1% PEG3350, pH 6.0 with varying concentrations of mannitol and sucrose as shown in Table 2.

Table 2

Formulation	Mannitol	Sucrose
T	5%	0.5%
A	5%	1%
B	5%	2%
C	5%	3%
D	4%	3%
E	3%	3%
F	--	5%

1.6-ml aliquots of the solutions were placed in vials and lyophilized under the conditions shown in Table 3. For subsequent analysis, lyophilized samples were reconstituted with water where necessary. Visual observations of lyophilized and reconstituted samples are shown in Table 4.

Table 3

Formulation	Freezing & Annealing	Primary Drying	Secondary Drying
T	0.5°C/min to 5°C, hold 0.5 hr 0.25°C/min to -50°C, hold 2 hr 0.25°C/min to -30°C, hold 3 hr 0.25°C/min to -50°C, hold 2 hr	0.5 °C/min to -10°C, hold 16 hr 60 mTorr	0.2 °C/min to 30°C, hold 24 hr 60 mTorr
A, B, C, D, E, F	0.5°C/min to 5°C, hold 2 hr 0.5°C/min to -50°C, hold 2 hr 0.25°C/min to -20°C, hold 2 hr 0.25°C/min to -25°C, hold 2 hr 0.25°C/min to -50°C, hold 2 hr	0.5 °C/min to -30 °C, hold 10 hr 0.5 °C/min to -25 °C, hold 10 hr 0.5 °C/min to -20 °C, hold 10 hr 0.5 °C/min to -15 °C, hold 10 hr 60 mTorr	0.5°C/min to 25°C, hold 24hr 0.5°C/min to 30°C, hold 8 hr 0.5°C/min to 20°C, hold 6 hr 60 mTorr

Table 4

Formulation	Lyophilized Solid	Reconstituted Solution
T	White cake	Clear
A	White cake	Clear
B	White cake	Clear
C	White cake	Clear
D	White cake	Clear
E	White semi-collapsed cake or white powder	Clear
F	White powder	Clear

Example 2

Formulations A, B, C, D, E, and F of rhThrombin were analyzed for the presence of aggregates/precipitants in solution. Pre- and post-lyophilization samples were analyzed by Right Angle Light Scattering (RALS) using a continuous spectrofluorometer (QUANTAMASTER QM4, Photon Technology International, Inc., Lawrenceville, NJ). The excitation and emission wavelengths were both set to 320 nm. Samples were loaded in a quartz vial of 1-cm pathlength. The slit widths were adjusted to 2 nm, and signals were collected at 1 point/sec for 60 seconds. The applied current was 75 watts. The reported values (see Fig. 1) were the average counts recorded over 60 seconds. Significant light scattering consistent with aggregation/precipitation after reconstitution was found in Formulation A. Formulations E and F did not produce an acceptable cake upon lyophilization.

Example 3

Recovery of rhThrombin after lyophilization was measured using reverse-phase chromatography (RP-HPLC). Size-exclusion chromatography (SE-HPLC) was used to determine the percentage of soluble aggregate in test samples. Formulations A, B, C, D, E, and F were analyzed prior to lyophilization and after lyophilization and reconstitution.

For RP-HPLC, test samples and reference standard (rhThrombin, 1 mg/mL in dilution buffer (20 mM sodium phosphate, 140 mM NaCl, pH 7.0)) were filtered using centrifugal filter units (0.22  $\mu$ m) (SPIN-X; Corning Life Sciences, Action, Mass.) at 14,000 RPM (20,800 RCF). A standard curve range from 5 to 40  $\mu$ g was employed with samples injected at approximately 90% of the upper limit of quantification. Purity values were determined based on the area of the main peak relative to the total integrated peak area. Analysis was performed using an HPLC system (1100 Series; Agilent Technologies, Palo Alto, California) configured with:

- Binary pump with seal wash kit (G1312A)

- Four channel solvent degasser (G1322A)
- Thermostatted autosampler (G1329A/G1330A)
- Extended volume upgrade kit utilizing a 900  $\mu\text{L}$  syringe (G1363A)
- Thermostatted column compartment with two heat exchangers (G1316A)
- Column switching upgrade kit (G1353A)
- Diode-array detector (G1315B) with 10 mm/13  $\mu\text{L}$  flow cell
- Agilent CHEMSTATION Software (Revision A.08.03)

For SE-HPLC, test samples and reference standard were filtered using centrifugal filter units (0.22  $\mu\text{m}$ ) at 14,000 RPM (20,800 RCF). A standard curve range from 5.1 to 102  $\mu\text{g}$  was utilized, with samples injected at approximately 90% of the upper limit of quantification. The percentage of aggregates was determined based on the area of the detected peaks preceding the main rhThrombin peak relative to the total integrated peak area. Analysis was performed using an HPLC system as disclosed above.

These analyses showed an increase in recovery of rhThrombin after lyophilization with increasing sucrose ( $\geq 3\%$ ) content as shown in Tables 5 and 6. Data are presented as rhThrombin concentration (mg/ml). The observation that increases in soluble aggregate did not necessarily correlate with lower recovery is consistent with the formation of insoluble rhThrombin aggregate during lyophilization.

Table 5

RP-HPLC						
Formulation:	A	B	C	D	E	F
Pre-lyophilization:	1.03	1.06	1.02	1.04	1.03	1.04
Reconstituted:	0.91	0.92	0.93	0.94	0.96	0.96
% Recovered	88.3%	86.8%	91.2%	90.4%	93.2%	92.3%

Table 6

% soluble aggregate by SE-HPLC						
Formulation	A	B	C	D	E	F
Pre-lyophilization	0.5	0.5	0.5	0.5	0.6	0.6
Post-lyophilization	0.6	0.7	0.7	0.7	0.5	0.5

Example 4

Storage stability of rhThrombin formulations was studied over a 6-month period. Lyophilized samples of formulations A, B, C, D, E, and F were stored at 40°C and at 25°C and analyzed for content (thrombin concentration) by RP-HPLC at 0, 1, 2, 3, and 6 months as disclosed above. At both temperatures, formulation A, upon reconstitution, became turbid after two months (Table 7). Formulations E and F produced unacceptable cakes and were not analyzed after the 0-month time point. Stability, expressed as % recovery, is shown in Tables 8 and 9.

Table 7

Temperature	Time	T	A	B	C	D
-20°C	0	Clear	Clear	Clear	Clear	Clear
	1 month	Clear	Clear	Clear	Clear	Clear
	2 months	Clear	Clear	Clear	Clear	Clear
	3 months	Clear	Clear	Clear	Clear	Clear
	6 months	Clear	Clear	Clear	Clear	Clear
5°C	1 week	Slightly turbid	Clear	Clear	Clear	Clear
	2 week	Clear	Clear	Clear	Clear	Clear
	1 month	Clear	Clear	Clear	Clear	Clear
	2 months	Clear	Clear	Clear	Clear	Clear
	3 months	Clear	Clear	Clear	Clear	Clear
	6 months	Clear	Clear	Clear	Clear	Clear
25°C	1 week	Slightly turbid	Clear	Clear	Clear	Clear
	2 week	Slightly turbid	Clear	Clear	Clear	Clear
	1 month	Slightly turbid	Clear	Clear	Clear	Clear
	2 months	Clear	Slightly turbid	Clear	Clear	Clear
	3 months	Slightly turbid	Slightly turbid	Clear	Clear	Clear
	6 months	Clear	Slightly turbid	Clear	Clear	Clear
40°C	1 week	Slightly turbid	Not tested	Not tested	Not tested	Not tested
	2 week	Slightly turbid	Not tested	Not tested	Not tested	Not tested
	1 month	Slightly turbid	Clear	Clear	Clear	Clear
	2 months	Not tested	Slightly turbid	Clear	Clear	Clear
	3 months	Not tested	Slightly turbid	Clear	Clear	Clear
	6 months	Not tested	Slightly turbid	Clear	Clear	Clear

Table 8

% recovery of thrombin during storage at 40 °C

Formulation	Months						
	0	0.25	0.5	1	2	3	6
T	100	87.5	83.3	84.4	n.t.	n.t.	n.t.
A	100	n.t.	n.t.	95.6	94.5	92.3	90.1
B	100	n.t.	n.t.	96.7	98.9	95.7	92.4
C	100	n.t.	n.t.	96.8	95.7	93.5	91.4
D	100	n.t.	n.t.	97.9	97.9	95.7	93.6

n.t. = not tested.

Table 9

% recovery of thrombin during storage at 25 °C

Formulation	Months					
	0	1	2	3	6	12
Tox	100	97.9	93.8	94.8	94.8	93.4
A	100	101	98.9	98.9	95.6	96.7
B	100	100	100	97.8	95.7	97.8
C	100	98.9	100	96.8	n.t.	100
D	100	98.9	98.9	97.9	97.9	97.9

\*n.t. = not tested.

Example 5

Lyophilized samples of formulations A, B, C, D, E, and F were stored at 40°C and at 25°C and analyzed at 0, 1, 2, 3, and 6 months for the presence of high molecular weight impurities (soluble aggregate) by SE-HPLC as disclosed above. Results, shown in Table 10, indicated that in formulation T (containing the least amount of sucrose), increases in soluble aggregate were detected after one week. However, in formulations containing 1% or more sucrose, significant increases were not found after six months. Formulations E and F produced unacceptable cakes and were not analyzed after the 0-month time point.

Table 10  
% aggregates; storage at 40°C

Formulation	Months						
	0	0.25	0.5	1	2	3	6
T	0.3	1.1	1.1	1.1	n.t.	n.t.	n.t.
A	0.6	n.t.	n.t.	0.7	0.8	0.6	0.8
B	0.7	n.t.	n.t.	0.6	0.6	0.5	0.9
C	0.7	n.t.	n.t.	0.6	0.6	0.6	0.6
D	0.7	n.t.	n.t.	0.6	0.6	0.6	0.7

n.t. = not tested.

Table 11  
% aggregates; storage at 25°C

Formulation	Months					
	0	1	2	3	6	12
T	0.3	0.4	0.5	0.5	0.7	0.5
A	0.6	0.5	0.5	0.5	0.6	0.6
B	0.7	0.8	0.7	0.6	0.8	0.6
C	0.7	0.8	0.8	0.6	0.6	0.6
D	0.7	0.7	0.6	0.6	0.7	0.6

#### Example 6

Lyophilized samples of formulations A, B, C, and D were stored for thirteen weeks and six months at -20°C, 5°C, 25°C and 40°C, then analyzed for RALS as disclosed in Example 2, above. Increases in light scattering measured by RALS are associated with the presence of insolubles and/or soluble aggregates. Results, shown in Tables 12 and 13 (as counts per second), and presented graphically in Figs. 2 and 3, indicated that Formulations B, C, and D were more stable than Formulation A with respect to aggregation during storage under all test conditions. Due to minor instrument variations between the 13-week and 6-month time points, the absolute numbers of two time points should not be compared; the RALS data should be compared only among the formulations tested at same time.

Table 12  
13 weeks

Storage Temperature	Formulation			
	A	B	C	D
-20°C	870,148.3	220,321.1	174,001.5	175,358.3
5°C	917,635.8	209,274.9	298,378.7	183,227.1
25°C	1,442,075	243,777.1	160,173.6	211,948.2
40°C	3,746,816	1,196,508	1,245,188	1,049,673

Table 13  
6 Months

Storage Temperature	Formulation			
	A	B	C	D
-20C	377158.2	72443.12	74719.25	92018.51
5C	438323.8	100675.1	100185.1	76518.18
25C	705247.2	175997.6	134422.6	92118.5
40C	1824263	438520.7	455521.4	446050.9

Example 7

Lyophilized samples of formulations A, B, C, and D were analyzed by Fourier transform infrared (FTIR) spectroscopy to compare the secondary structure of rhThrombin in the lyophilized state. Infrared spectra were obtained with a FTIR spectrometer (FTLA2000-104, ABB Inc., Norwalk, Conn.). The lyophilized samples were mixed with potassium bromide (KBr) (approximate ratio 1:100 rhThrombin:KBr) and pressed to form pellets. The spectra were collected in single-beam transmission mode. To reduce the interferences from excipients and water vapor, the spectra of placebo and water vapor were subtracted from the protein spectra using analytical software (BOMEN-GRAMS/32 AI (Version 6.01); ABB Inc.). The second derivative spectra were created using the Savitzky-Golay function of second degree. The rhThrombin formulations were compared in the amide I region (1700 -1600  $\text{cm}^{-1}$ ). As shown in Fig. 4, similar spectra were obtained for formulations B, C, and D, with formulation A giving a different spectrum.

Three replicates of formulation A were then tested by FTIR as disclosed above. The resulting spectra were essentially identical.

Formulations A, B, C, and D were then compared to a formulation containing 5% mannitol and 0.5% sucrose (Formulation T). FTIR was carried out as disclosed above. As shown in Fig. 5, Formulations B, C, and D gave similar spectra, while the spectra of formulations containing 5% mannitol and either 1% sucrose or 0.5% sucrose indicated a different spectra. The increases in RALS observed after lyophilization when formulations containing  $\leq 1\%$  sucrose are compared to those

containing  $\geq 2\%$  sucrose are consistent with these findings (Example 6). These data suggest that thrombin is partially unfolded in formulations containing  $\leq 1\%$  sucrose (leading to increased insolubles upon reconstitution), whereas, the secondary structure of thrombin is stabilized in the presence of  $\geq 2\%$  sucrose during lyophilization.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.



## CLAIMS

What is claimed is:

1. A composition consisting essentially of:

0.1 mg/mL to 5.0 mg/mL thrombin;

2% to 4% (w/v) sucrose;

3.5% to 5% (w/v) mannitol;

50 mM to 300 mM NaCl;

0-5mM CaCl<sub>2</sub>;

0.03% to 1% (w/v) of a surfactant or high-molecular-weight polyethylene glycol; and  
a physiologically acceptable buffer,

in aqueous solution at pH 5.7 - 7.4, wherein the concentration of the buffer is selected to provide approximately physiological pH upon application of the composition in a surgical setting and wherein the ratio of mannitol: sucrose is greater than 1:1 but not greater than 2.5:1 (w/w).

2. The composition of claim 1 wherein the ratio of mannitol to sucrose is 1.33:1 (w/w).

3. The composition of claim 1 wherein the molar ratio of sucrose:thrombin is at least 700:1.

4. The composition of claim 1 wherein the molar ratio of sucrose:thrombin is at least 2000:1.

5. The composition of claim 1 wherein the thrombin is human thrombin.

6. The composition of claim 1 wherein the thrombin is recombinant human thrombin.

7. The composition of claim 1 wherein the thrombin concentration is 0.5 - 3.0 mg/ml.

8. The composition of claim 1 wherein the thrombin concentration is 1 mg/ml.

9. The composition of claim 1 wherein the sucrose concentration is 3% (w/v).

10. The composition of claim 1 wherein the mannitol concentration is 4% (w/v).
11. The composition of claim 1 wherein the sucrose concentration is 3% (w/v) and the mannitol concentration is 4% (w/v).
12. The composition of claim 1 wherein the surfactant or high-molecular-weight polyethylene glycol is PEG 3350.
13. The composition of claim 12 wherein the concentration of PEG 3350 is 0.1% (w/v).
14. The composition of claim 1 wherein the pH is 6.0.
15. The composition of claim 1 wherein the buffer is selected from the group consisting of histidine, citrate, phosphate, Tris, and succinate buffers.
16. The composition of claim 1 wherein the buffer is histidine buffer.
17. The composition of claim 16 wherein the histidine buffer concentration is 2.0-10 mM.
18. The composition of claim 1 wherein the buffer is 5 mM histidine pH 6.0.
19. The composition of claim 1, consisting essentially of:
  - 0.1 mg/mL to 5.0 mg/mL thrombin;
  - 2% to 4% (w/v) sucrose;
  - 3.5% to 5% (w/v) mannitol;
  - 100 mM to 200 mM NaCl;
  - 1 mM to 5 mM CaCl<sub>2</sub>;
  - 0.03% to 1% (w/v) PEG3350; and
  - 2 mM to 10 mM histidinein aqueous solution at pH 5.5 - 6.5, wherein the ratio of mannitol: sucrose is greater than 1:1 but not greater than 2.5:1 (w/w).
20. The thrombin composition of claim 19, consisting essentially of:

1 mg/ml thrombin;  
3% (w/v) sucrose;  
4% (w/v) mannitol;  
4 mM CaCl<sub>2</sub>;  
0.1% (w/v) PEG3350;  
150 mM NaCl; and  
5 mM histidine

in aqueous solution at pH 6.0.

21. A method of preparing a lyophilized thrombin composition comprising:  
providing a composition according to any one of claims 1 to 20; and  
lyophilizing the aqueous solution to form a lyophilized thrombin composition.

22. The method of claim 21 wherein, within the providing step, the composition consists essentially of:

0.1 mg/mL to 5.0 mg/mL thrombin;  
2% to 4% (w/v) sucrose;  
3.5% to 5% (w/v) mannitol;  
100 mM to 200 mM NaCl;  
1 mM to 5 mM CaCl<sub>2</sub>;  
0.03% to 1% (w/v) PEG3350; and  
2 mM to 10 mM histidine

in aqueous solution at pH 5.5 - 6.5, wherein the ratio of mannitol: sucrose is greater than 1:1 but not greater than 2.5:1 (w/w).

23. A lyophilized thrombin composition prepared by the method of claim 21.

24. The composition of claim 23 contained in a sealed container having a label affixed to an exterior surface thereof.

25. The composition of claim 24, which contains from 2,500 to 20,000 NIH units of thrombin.

26. A kit comprising the composition of claim 24 and a second sealed container containing a diluent.

27. The kit of claim 26 wherein the diluent is normal saline.

28. A lyophilized composition prepared from a solution consisting essentially of:

0.1 mg/mL to 5.0 mg/mL thrombin;

2% to 4% (w/v) sucrose;

3.5% to 5% (w/v) mannitol;

50 mM to 300 mM NaCl;

0-5mM CaCl<sub>2</sub>;

0.03% to 1% (w/v) of a surfactant or high-molecular-weight polyethylene glycol; and  
a physiologically acceptable buffer,

in aqueous solution at pH 5.7 - 7.4, wherein the concentration of the buffer is selected to provide approximately physiological pH upon application of the composition in a surgical setting and wherein the ratio of mannitol: sucrose is greater than 1:1 but not greater than 2.5:1 (w/w).

29. The lyophilized composition of claim 28 wherein the ratio of mannitol to sucrose is 1.33:1 (w/w).

30. The lyophilized composition of claim 28 wherein the molar ratio of sucrose:thrombin is at least 700:1.

31. The lyophilized composition of claim 28 wherein the molar ratio of sucrose:thrombin is at least 2000:1.

32. The lyophilized composition of claim 28 wherein the thrombin is human thrombin.

33. The lyophilized composition of claim 28 wherein the thrombin is recombinant human thrombin.

34. The lyophilized composition of claim 28 wherein the thrombin concentration is 0.5 - 3.0 mg/ml.
35. The lyophilized composition of claim 28 wherein the thrombin concentration is 1 mg/ml.
36. The lyophilized composition of claim 28 wherein the sucrose concentration is 3% (w/v).
37. The lyophilized composition of claim 28 wherein the mannitol concentration is 4% (w/v).
38. The lyophilized composition of claim 28 wherein the sucrose concentration is 3% (w/v) and the mannitol concentration is 4% (w/v).
39. The lyophilized composition of claim 28 wherein the surfactant or high-molecular-weight polyethylene glycol is PEG 3350.
40. The lyophilized composition of claim 39 wherein the concentration of PEG 3350 is 0.1% (w/v).
41. The lyophilized composition of claim 28 wherein the pH is 6.0.
42. The lyophilized composition of claim 28 wherein the buffer is selected from the group consisting of histidine, citrate, phosphate, Tris, and succinate buffers.
43. The lyophilized composition of claim 28 wherein the buffer is histidine buffer.
44. The lyophilized composition of claim 43 wherein the histidine buffer concentration is 2.0-10 mM.
45. The lyophilized composition of claim 28 wherein the buffer is 5 mM histidine pH 6.0.
46. The lyophilized composition of claim 28, consisting essentially of:  
0.1 mg/mL to 5.0 mg/mL thrombin;

2% to 4% (w/v) sucrose;  
3.5% to 5% (w/v) mannitol;  
100 mM to 200 mM NaCl;  
1 mM to 5 mM CaCl<sub>2</sub>;  
0.03% to 1% (w/v) PEG3350; and  
2 m.M. to 10 mM histidine

in aqueous solution at pH 5.5 - 6.5, wherein the ratio of mannitol:sucrose is greater than 1:1 but not greater than 2.5:1 (w/w).

47. The lyophilized composition of claim 46, consisting essentially of:

1 mg/ml thrombin;  
3% (w/v) sucrose;  
4% (w/v) mannitol;  
150 mM NaCl;  
4 mM CaCl<sub>2</sub>;  
0.1% (w/v) PEG3350; and  
5 inM histidine

in aqueous solution at pH 6.0.

48. The lyophilized composition of claim 28 contained in a sealed container having a label affixed to an exterior surface thereof.

49. The lyophilized composition of claim 48, which contains from 2,500 to 20,000 NIH units of thrombin.

50. A kit comprising the lyophilized composition of claim 28 and a second sealed container containing a diluent.

51. The kit of claim 50 wherein the diluent is normal saline.

52. The lyophilized composition of claim 28 wherein the ratio of mannitol to sucrose is 1.33:1 (w/w) and the concentration of sodium chloride is 75 mM to 250 mM.

53. The lyophilized composition of claim 28 wherein the ratio of mannitol to sucrose is 1.33:1 (w/w) and the concentration of sodium chloride is 100 mM to 200 mM.

54. The lyophilized composition of claim 28 wherein the ratio of mannitol to sucrose is 1.33:1 (w/w) and the concentration of sodium chloride is 150 mM.

55. Use of the composition of any one of claims 1 to 20 as a hemostatic agent or a component of a tissue adhesive.

56. Use of the lyophilized composition of any one of claims 28 to 49 or 52 to 54, for reconstitution of a hemostatic agent or a component of a tissue adhesive.

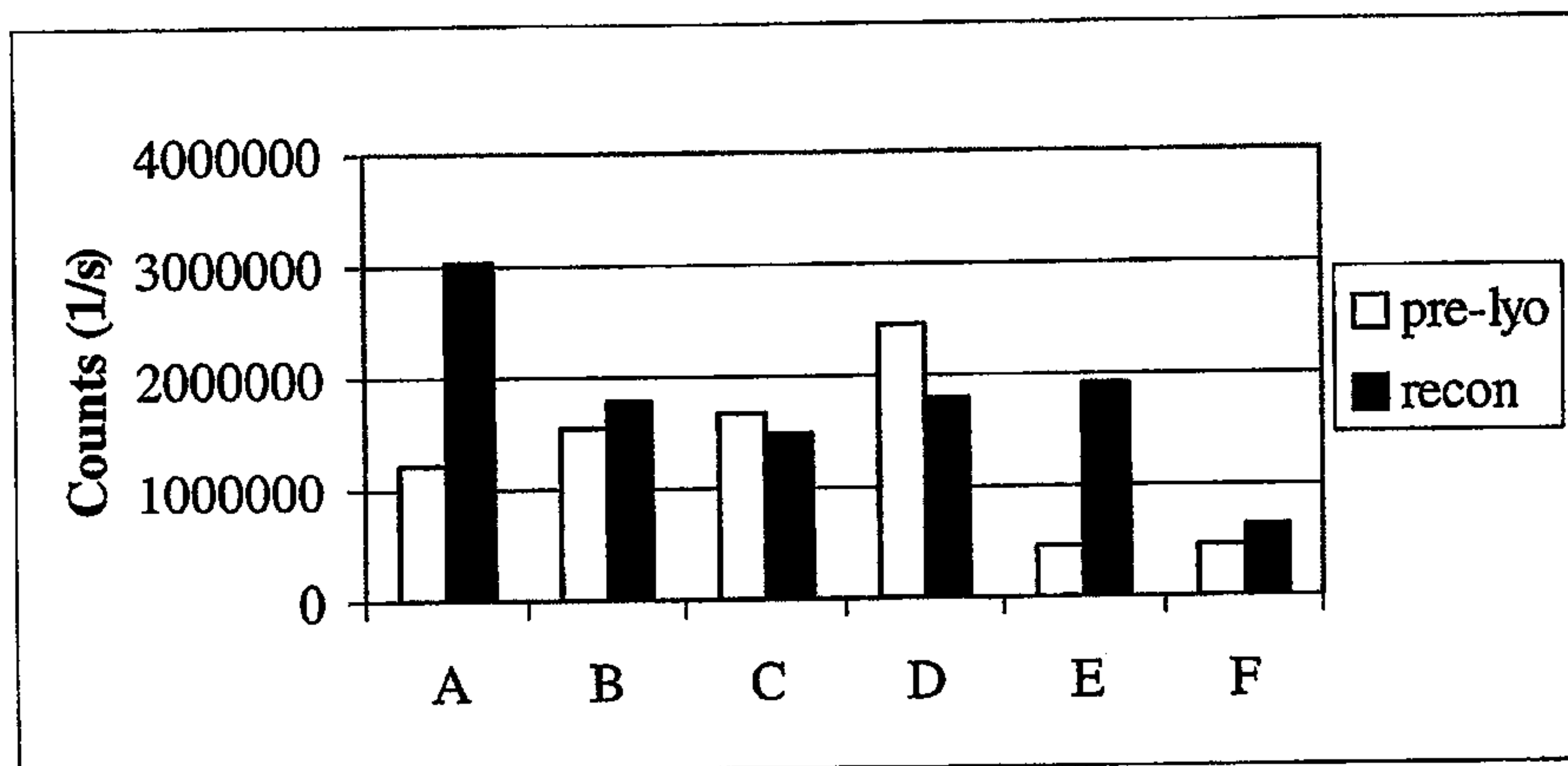


Fig. 1



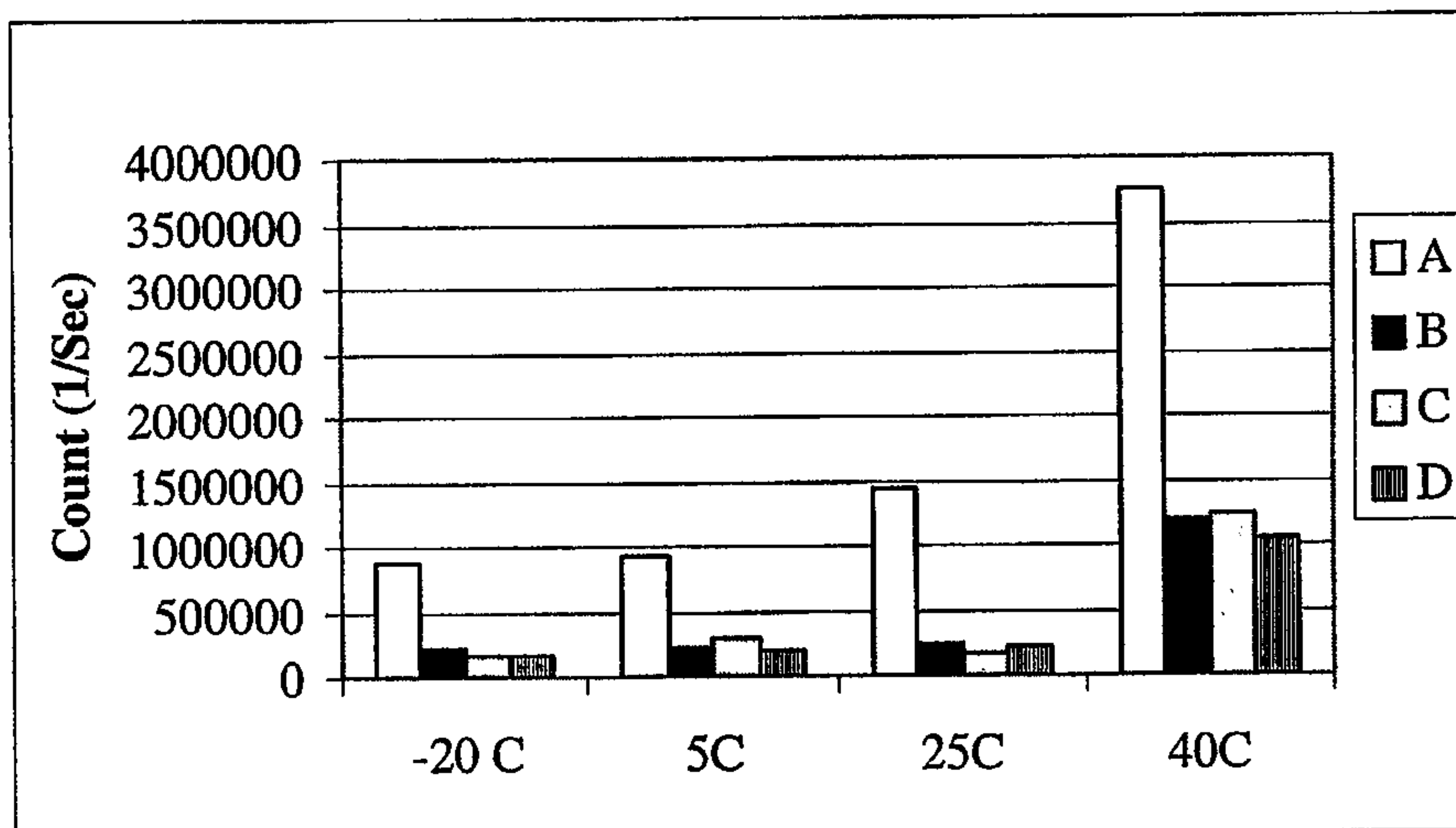


Fig. 2

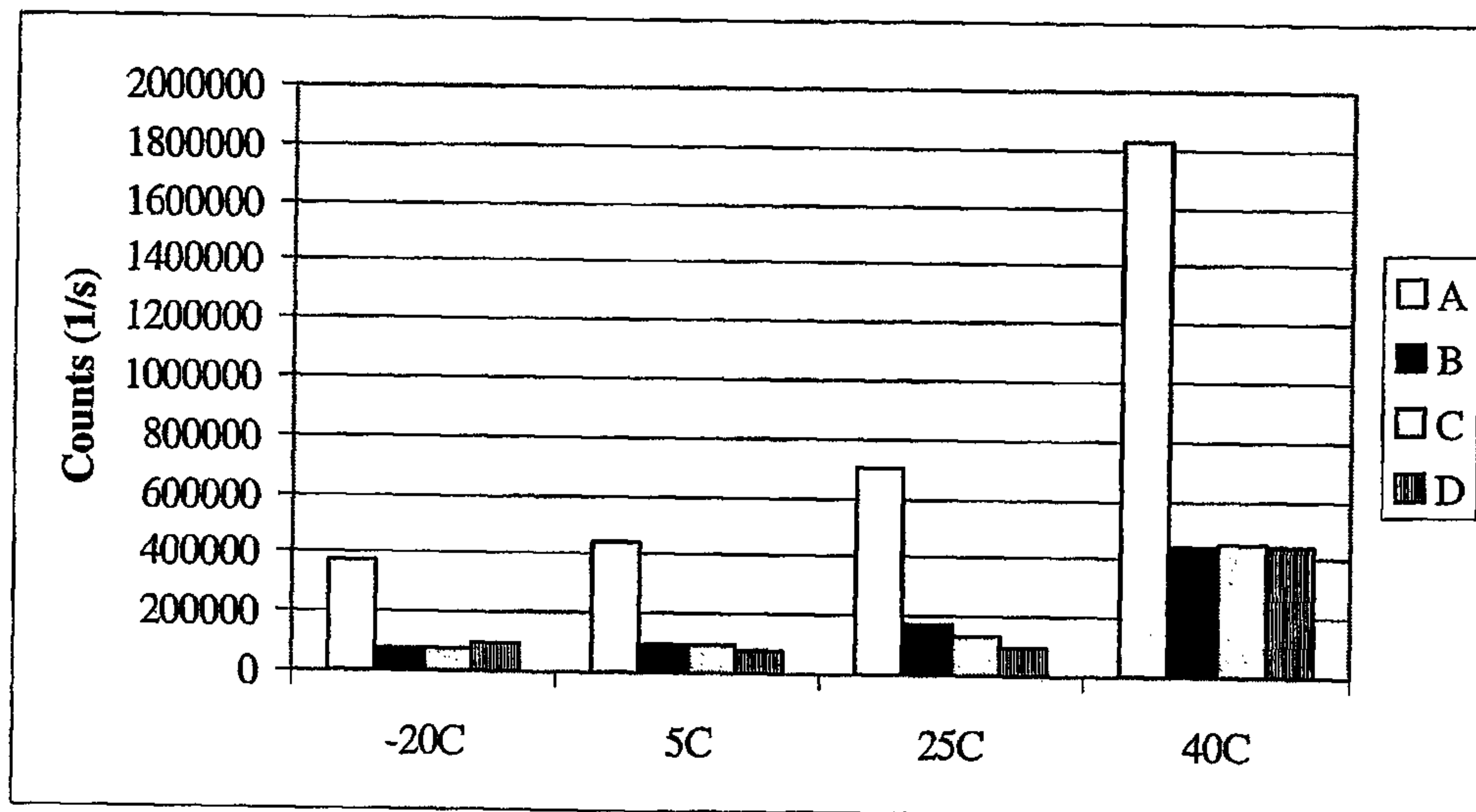


Fig. 3

Fig. 4

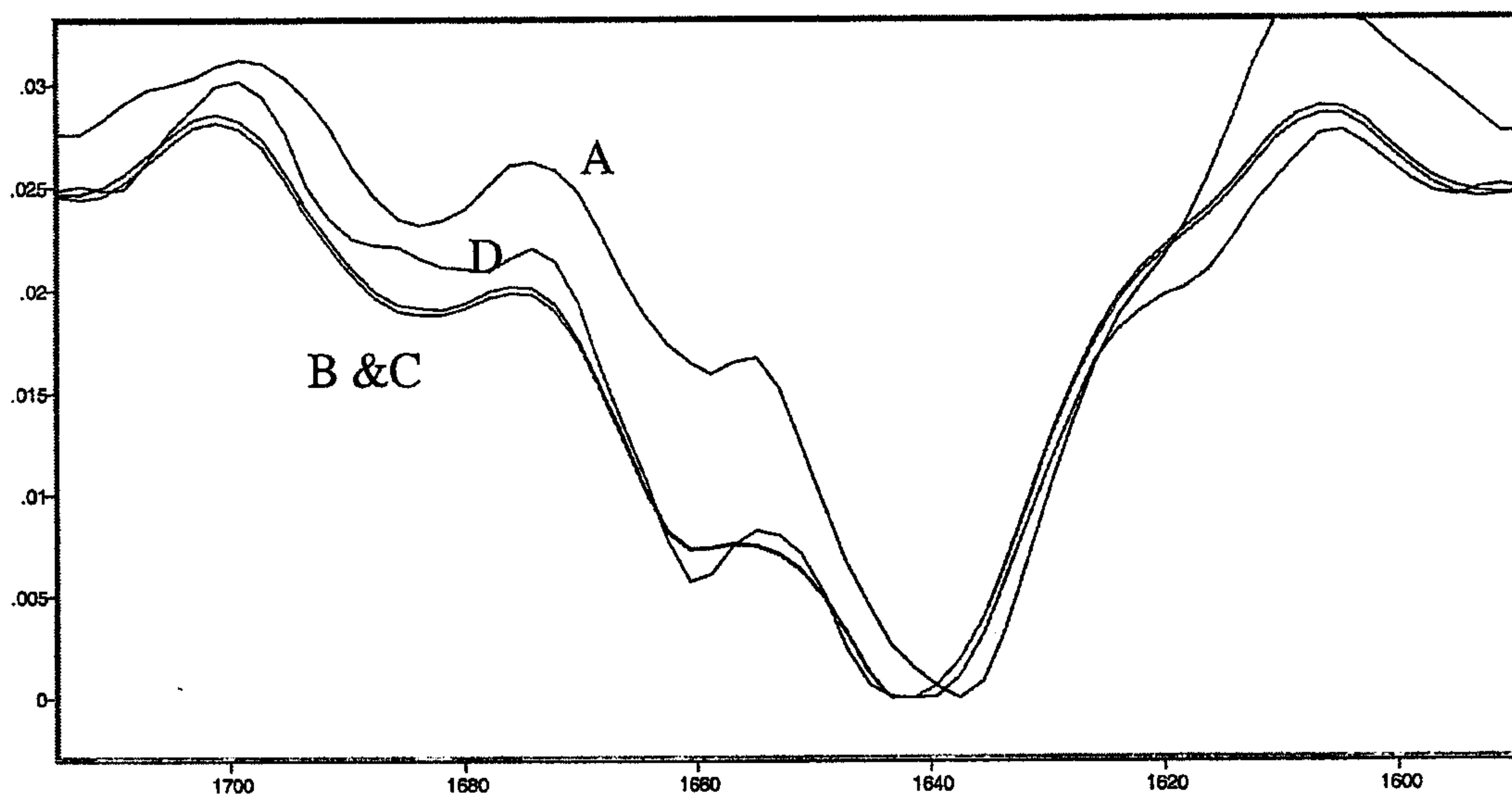


Fig. 5

