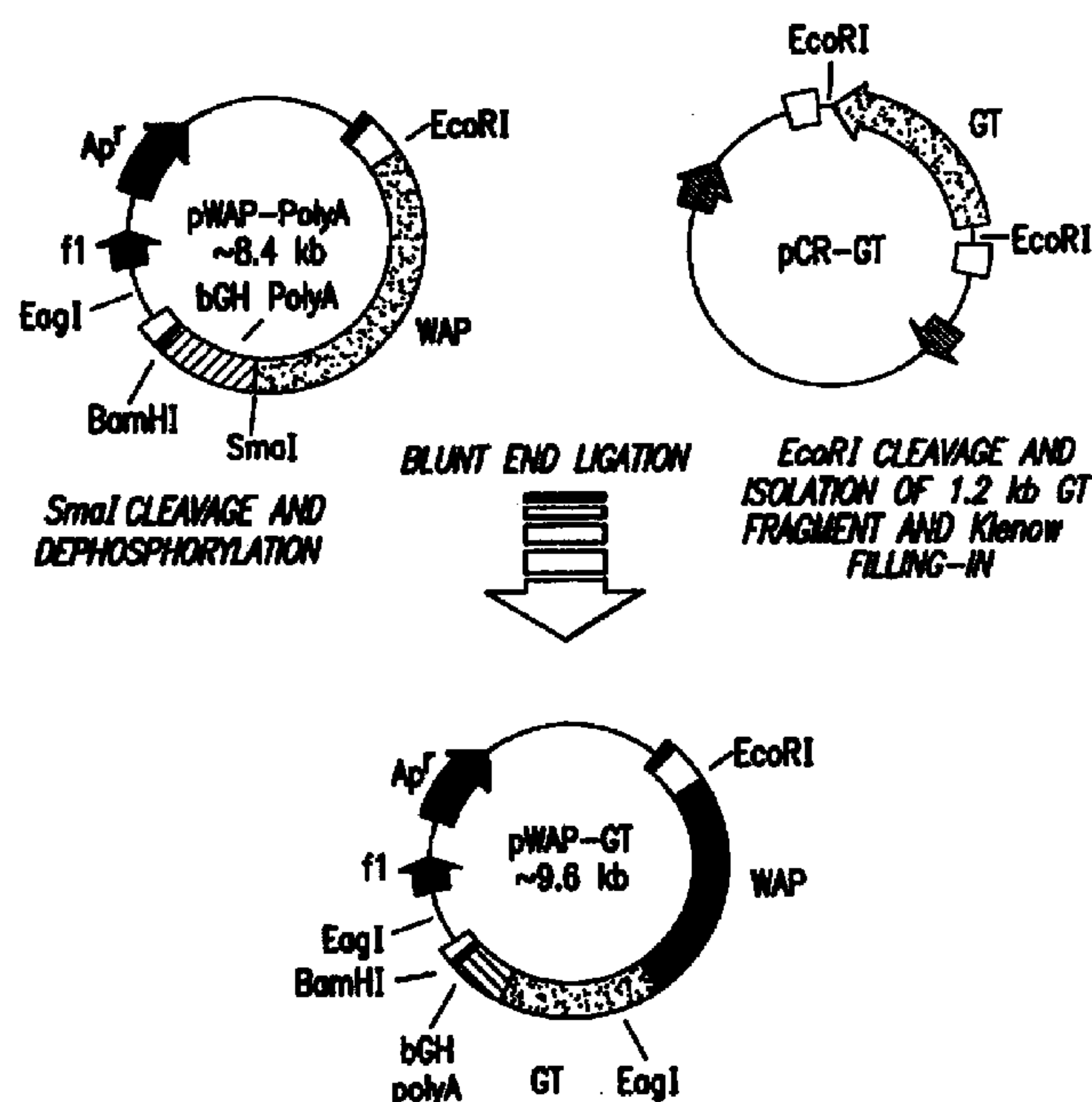




- (72) PRIETO, PEDRO A., US
(72) KOPCHICK, JOHN J., US
(72) CUMMINGS, RICHARD D., US
(72) PIERCE, JAMES M., US
(72) SMITH, DAVID F., US
(72) MOREMEN, KELLEY W., US
(71) ABBOTT LABORATORIES, US
(71) OHIO UNIVERSITY, US
(71) UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC., US
(51) Int.Cl.⁷ C12N 15/00, A23C 9/20, C12N 9/10, A01K 67/027
(30) 1997/09/05 (08/925,834) US
(54) **MAMMIFERES TRANSGENIQUES PRODUISANT DES
OLIGOSACCHARIDES DANS LEUR LAIT**
(54) **TRANSGENIC MAMMALS WHICH PRODUCE
OLIGOSACCHARIDES IN THEIR MILK**



(57) La présente invention concerne des procédés permettant d'obtenir des mammifères transgéniques non humains produisant plusieurs oligosaccharides et glycoconjugués dans leur lait. En outre, l'invention concerne les mammifères eux-mêmes, le lait qu'ils produisent, des compositions renfermant le lait, des fractions de lait et les oligosaccharides purifiés, ainsi que les glycoconjugués présents dans le lait.

(57) The subject invention relates to methods of producing non-human transgenic mammals which produce various oligosaccharides and glycoconjugates in their milk. Additionally, the subject invention relates to the mammals themselves, the milk which they produce, compositions comprising the milk, fractions of the milk, and the purified oligosaccharides, as well as glycoconjugates, present in the milk.



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/00, 9/10, A01K 67/027, A23C 9/20	A1	(11) International Publication Number: WO 99/11773 (43) International Publication Date: 11 March 1999 (11.03.99)
(21) International Application Number: PCT/US98/18383 (22) International Filing Date: 4 September 1998 (04.09.98) (30) Priority Data: 08/925,834 5 September 1997 (05.09.97) US (71) Applicant: ABBOTT LABORATORIES [US/US]; CHAD 0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US). (72) Inventors: PRIETO, Pedro, A.; 1644 Crusoe Drive, West Worthington, OH 43235 (US). KOPCHICK, John, J.; 4 Orchard Lane, Athens, OH 45701 (US). CUMMINGS, Richard, D.; 5215 North Santa Fe, Edmond, OK 73003 (US). PIERCE, James, M.; 225 Garland Hill Drive, Athens, GA 30605 (US). SMITH, David, F.; 205 Pendleton Drive, Athens, GA 30606 (US). MOREMEN, Kelley, W.; 745 Kings Road, Athens, GA 30606 (US). (74) Agents: BECKER, Cheryl, L. et al.; Abbott Laboratories, CHAD 0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: TRANSGENIC MAMMALS WHICH PRODUCE OLIGOSACCHARIDES IN THEIR MILK (57) Abstract The subject invention relates to methods of producing non-human transgenic mammals which produce various oligosaccharides and glycoconjugates in their milk. Additionally, the subject invention relates to the mammals themselves, the milk which they produce, compositions comprising the milk, fractions of the milk, and the purified oligosaccharides, as well as glycoconjugates, present in the milk. SmaI CLEAVAGE AND DEPHOSPHORYLATION EcoRI CLEAVAGE AND ISOLATION OF 1.2 kb GT FRAGMENT AND Klenow FILLING-IN BLUNT END LIGATION		

TRANSGENIC MAMMALS WHICH PRODUCE OLIGOSACCHARIDES IN THEIR MILK

The subject application is a continuation-in-part of
5 pending U.S. patent application serial no. 08/208,889, filed
March 9, 1994, pending U.S. patent application serial no.
08/715,259, filed September 16, 1996, which is a continuation of
abandoned U.S. patent application serial no. 08/209,132, filed
on March 9, 1994, pending U.S. patent application serial no.
10 08/434,151, filed May 2, 1995, which is a divisional of pending
U.S. patent application serial no. 08/209,132, filed March 9,
1994, and pending U.S. patent application serial no. 08/433,271,
filed on May 2, 1995. which is a divisional of pending U.S.
patent application serial no. 08/209,122, all of which are
15 hereby incorporated in their entirety by reference.

BACKGROUND OF THE INVENTIONTechnical Field

20 The subject invention relates to methods of producing non-
human transgenic mammals which produce various oligosaccharides
in their milk. Additionally, the subject invention relates to
the generated mammals, the milk which these mammals produce,
compositions comprising the milk, fractions of the milk, and the
25 purified oligosaccharides, as well as glycoproteins, present in
the milk.

Background Information

In the past, several individuals have achieved transgenic
30 expression of primary gene products (i.e., proteins) in the milk
of transgenic mammals (U.S. Patent No. 5,322,775, Wall et al.,
Proc. Natl. Acad. Sci. USA 88:1696-1700 (1991) and Krimpenfort,
P., *Cancer Detection and Prevention* 17(2):301-305 (1993)). Such
expression was carried out in order to produce large quantities
35 of the heterologous proteins in milk (Velander et al.,
Scientific American, January 1997, pp.70-74).

Recently, it was also demonstrated that expression of secondary transgenic products, for example, oligosaccharides, glycoproteins and glycolipids is, in fact, possible (Prieto et al., *Journal of Biological Chemistry* 49:29515-29519 (1995)).

5 The experiments carried out by Prieto et al. advanced the concept of using the mammary glands of transgenic mammals as bioreactors and not solely as an alternative protein synthesis system.

10 Most of the experiments referenced in the above articles were carried out by expression of heterologous transgenes. One reason for such expression is because homologous genes, which are transgenically expressed, may cause difficulties, due to conflicts with internal protein synthesis of mammary gland

15 development (Burdon et al., *Mechanisms of Development* 36:64-67 (1991)). Another reason why heterologous transgenes are utilized is that most of the experiments which have been carried out are designed for the production of human biological products.

20

The synthesis of secondary gene products, such as milk oligosaccharides, depends primarily on the expression of glycosyltransferases during lactation. Additionally, there are many benefits to the production of such oligosaccharides. For

25 example, in a transgenic system for the synthesis of oligosaccharides, only catalytic amounts of glycosyltransferases are necessary. Also, the origin of the transgene may not be relevant as long as the secondary gene product or products are the desired one or ones.

30

It must also be noted that non-human mammals have a variety of glycosyltransferases which have counterparts in the human enzyme repertoire (Thurin et al., *The Journal of Biological Chemistry* 265(12):7055-7061 (1990)). Some of these transferases

35 have been cloned from the tissues of non-human mammals, for example, mice (Larsen et al., *Proc. Natl. Acad. Sci. USA* 86:8227-8231 (1989)). Thus, transgenic expression of homologous

glycosyltransferases in the mammary glands of non-human mammals represents a means of producing oligosaccharides, in addition to oligosaccharides produced using heterologous glycosyltransferases. Expression of both such enzyme types, as well as the oligosaccharides which are created by the action of such enzymes, and glycoproteins related thereto, are considered to fall within the scope of the present invention.

The oligosaccharides described above may be added, for example, to infant formula so that the formula-fed infant may receive the same benefits as the breast fed infant. For example, it is possible that the oligosaccharides may provide resistance to infection by pathogenic bacteria (Prieto et al., *supra*). Additionally, such oligosaccharides may be added to other compositions such as nutritional supplements or to pharmaceuticals. The aim of the present invention is to produce non-human transgenic mammals which produce such oligosaccharides, using either homologous or heterologous enzymes including, for example, glycosyltransferases. Such oligosaccharides may then be added to various compositions due to their beneficial properties.

Additionally, the presence of oligosaccharides and other glycoconjugates in the milk of transgenic animals may result in the acquisition of protection or resistance to viral, bacterial or fungal infections or toxins, by the offspring of such animals.

SUMMARY OF THE INVENTION

The present invention encompasses a method of synthesizing oligosaccharides and glycoproteins through transgenic expression of enzymes, for example, glycosyltransferases, during lactation. Furthermore, the invention also includes the production of oligosaccharide structures which are not present in nature. Such structures may be produced by transgenically expressing the appropriate eukaryotic or prokaryotic enzyme of interest, for

example, a glycosyltransferase, in a mammal. Thus, the present invention allows for the production of homologous, heterologous and novel structures in the milk of transgenic mammals.

5 More specifically, the present invention encompasses a method for producing a non-human, transgenic mammal whose somatic and germ cells contain at least one transgene. Expression of the transgene results in the production of oligosaccharides and glycoproteins in the milk of the mammal.

10 The method comprises the steps of: (a) preparing at least one transgene, (b) introducing the transgene or transgenes into a non-human, mammalian embryo, and transferring the resulting embryo into a recipient female, and then (c) identifying at least one female offspring. Expression of the transgene or

15 transgenes results in the production of at least one enzyme which then catalyzes the production of oligosaccharides and glycoproteins in the milk of the mammal. Each transgene itself comprises, in operable association, at least one expression regulatory sequence functional in mammary secretory cells, a DNA

20 sequence encoding a signal sequence functional in mammary secretory cells, and a DNA sequence encoding an enzyme. The non-human, transgenic mammal may be, for example, a mouse, a rat, a rabbit, a pig, a goat a sheep or a cow. The enzyme may be, for example, a glycosyltransferase such as a

25 fucosyltransferase, a galactosyltransferase, an acetylase, a glucoronyltransferase, a gluconylepimerase, a sialyltransferase, a mannosyltransferase, a sulfotransferase, a β -acetylgalactosaminyltransferase or a N-acetylglucosaminyltransferase. Additionally, the oligosaccharides may be, for

30 example, galactose α 1-3galactose β 1-4 glucose, 2'fucosyllactose, 3'fucosyllactose, lacto-N-neo-tetraose, lacto-N-tetraose, lacto-N-fucopentaose, a sialylated derivative of lacto-N-fucopentaose, lacto-N-fucopentaose II, a sialylated derivative of lacto-N-fucopentaose II, lacto-N-fucopentaose III,

35 a sialylated derivative of lacto-N-fucopentaose III, lacto-N-fucopentaose IV, a sialylated derivative of lacto-N-fucopentaose

IV, lacto-N-fucopentaose V, a sialylated derivative of lacto-N-fucopentaose V, lacto-N-di-fucopentaose I, a sialylated derivative of lacto-N-di-fucopentaose, lacto-N-di-fucopentaose II, a sialylated derivative of lacto-N-di-fucopentaose II, lacto-N-hexaose, a fucosylated derivative of lacto-N-hexaose, sialyltetrasaccharide a, a fucosylated derivative of sialyltetrasaccharide a, sialyltetrasaccharide b, a fucosylated derivation of sialyltetrasaccharide b, sialyltetrasaccharide c or a fucosylated derivative of sialyltetrasaccharide c.

10

Additionally, the present invention includes a non-human, transgenic mammal. The genome of the mammal comprises at least one DNA sequence encoding an enzyme operatively linked to a mammary gland-specific promoter. Expression of the DNA sequence or sequences results in the production of oligosaccharides and glycoproteins in the milk of the mammal. The mammal, enzyme and oligosaccharides may be those recited above.

Furthermore, the present invention includes a method of producing milk in a non-human, transgenic mammal. The method includes (a) preparing at least one transgene, (b) inserting the transgene or transgenes into the non-human, transgenic mammal, and (c) milking the non-human, transgenic mammal in order to obtain the milk. The milk comprises the oligosaccharides and glycoproteins. Each transgene comprises, in operable association, a promoter functional in mammary secretory cells and a DNA sequence encoding an enzyme. Expression of each transgene results in production of oligosaccharides and glycoproteins in the milk of the mammal. The mammal, enzyme and oligosaccharides may be, for example, those recited above.

Furthermore, the present invention encompasses a milk produced by a non-human, transgenic mammal. The genome of the non-human, transgenic mammal comprises at least one DNA sequence encoding an enzyme. The DNA sequence is operatively linked to a promoter. Expression of each DNA sequence results in the ultimate production of oligosaccharides and glycoproteins.

Again, the mammal, enzyme and oligosaccharides may be, for example, those recited above.

Moreover, the present invention also includes a fraction of the milk described above. This fraction may include, for example, one or more of the soluble oligosaccharides and/or glycoconjugates produced as a result of expression of the transgene or transgenes. Thus, a fraction of the milk may comprise one or more of the above components (i.e., oligosaccharides or glycoconjugates) or any combination thereof. Such a fraction may also include oligosaccharides naturally occurring in the milk of the transgenic mammal in combination with one or more of the oligosaccharides and/or glycoconjugates produced as a result of the presence of the transgene or transgenes.

Additionally, the present invention encompasses an oligosaccharide or glycoprotein purified from the milk described above, as well as a nutritional product containing the milk, a fraction thereof, or the oligosaccharides and/or glycoproteins, all of which have been described above. This nutritional product may be an infant formula in either liquid or solid form.

The present invention also includes a method of providing nutrition to patients. This method comprises administering to the patients the milk, fraction thereof, or oligosaccharides or glycoproteins, all described above, in an amount sufficient to effect the nutrition.

30

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1-6 relate to a method involving expression of a homologous enzyme (i.e., murine UDP-Gal β -D- α 1,3-galactosyltransferase).

Figure 1 illustrates the creation of the pWAP-GT construct. Figure 2 illustrates chromatographic analysis of galactose

α 1-3galactose β 1-4 glucose, a novel oligosaccharide produced by one of the methods of the present invention.

Figure 3 represents fluorophore assisted carbohydrate electrophoresis (FACE) analysis of galactose α 1-3galactose β 1-4 glucose.

Figure 4 represents FACE analysis of mouse milk spiked with galactose α 1-3galactose β 1-4 glucose.

Figure 5 illustrates an example of a negative transgenic, positive transgenic, and negative control mouse milk oligosaccharide profile, as analyzed using Dionex Method 2. The large peak eluting at 31-32 minutes is presumed to be the trisaccharide.

Figure 6 represents FACE analysis of the samples used in Figure 5. In particular, FACE analysis was carried out in order to verify that the "new" peak detected in Figure 5 was the trisaccharide. The band that runs concurrent with the G3 starch hydrolysate standard band is, indeed, the trisaccharide.

Figures 7-13 relate to a method involving expression of a heterologous enzyme (i.e., H- α -1,2-fucosyltransferase).

Figure 7 illustrates the High pH, Anion Exchange Chromatography (HPAEC) oligosaccharide profiles of two rabbit milk samples. Panel A is the milk oligosaccharide profile of a sample obtained from a control, non-transgenic animal. This sample contains lactose and only trace amounts of 2'-fucosyllactose (Fuc α 1-2Gal β 1-4Glc). Panel B is the profile of a sample obtained from a lactating rabbit whose genome contained a transgene encoding human H- α -1,2fucosyltransferase. This sample contains quantifiable 2'-fucosyllactose and has less than one-tenth of the lactose concentration present in the milk sample of the non-transgenic animal. Panel C is the profile obtained from the same sample used to generate the profile in Panel B but after the sample was spiked with 20 mg/liter of 2'-fucosyllactose.

Figure 8 represents the ion chromatographic profiles of

additional transgenic rabbit milk samples. Panel A is control milk, panel B is transgenic sample #2, panel C is transgenic sample #3, and panel D is transgenic sample #4. Only sample #4 (panel C) contained significant quantities of 2'-fucosyllactose (i.e., >50 mg/L). Sample #3 (panel B) exhibited a marked decrease in its lactose concentration.

Figure 9 illustrates Fluorophore Assisted Carbohydrate Electrophoresis (FACE) of milk oligosaccharides before and after digestion with α 1-2fucosyllactose from untreated and enzyme controlled rabbit milk, transgenic rabbit milk and an authentic standard. In particular, lane 1 represents authentic 2'-fucosyllactose standard, lane 2 represents untreated transgenic sample #5, lane 3 represents the fucosidase-treated transgenic sample #5, lane 4 represents an untreated, non-transgenic control sample, and lane 5 represents the fucosidase treated control sample. Lane 6 represents a set of linear glucose polymer standards where G2 represents two glucose units, and G3 represents three glucose units.

Figure 10 represents FACE of additional transgenic rabbit samples. In particular, lane 1 represents authentic 2'-fucosyllactose standard, lane 2 represents untreated transgenic sample #5, lane 3 represents an additional transgenic milk sample, lane 4 represents transgenic sample #4, lane 5 represents a non-transgenic control sample, lane 6 represents a set of linear glucose polymer standards, and lane 7 represents transgenic sample #3.

Figure 11 illustrates a Ulex Europaeus Agglutinin I (UEA-1) Western blot of transgenic and control rabbit milk proteins. Lane 1 represents a non-transgenic control sample, lane 2 represents transgenic sample #1, lane 3 represents transgenic sample #2, lane 4 represents the second non-transgenic control.

Figure 12 represents a UEA-1 Western blot of the additional rabbit milk protein samples. Lane 1 is from a non-transgenic control, lane 2 is transgenic sample #4, lane 3 is transgenic sample #3, lane 4 is a non-transgenic control sample, and lane 5 represents molecular weight standards.

Figure 13 illustrates a Coomassie stained SDS electrophoretogram in which rabbit milk proteins have been resolved. Lane 1 shows stained proteins from milk of a non-transgenic control, lane 2 is transgenic sample #3, lane 3 is transgenic sample #4, lane 4 is transgenic sample #5, and lane S Shows stained molecular weight standards.

Figures 14-16 relate to a method of expressing an enzyme (i.e., human fucosyltransferase IV) using heterologous lactogenically induced transgenic expression.

Figure 14 illustrates chromatographic profiles of milk oligosaccharide extracts. Panel A represents the control oligosaccharide extract, panel B represents the transgenic milk oligosaccharide extract, and panel C represents the spiked (i.e., 3-fucosyllactose) transgenic milk oligosaccharide extract.

Figure 15 represents the FACE results of a gel electrophoresis used to resolve fluorophore-labeled oligosaccharides. Lane 1 is a labelled starch hydrolyzate used as a molecular weight standard, lane 2 is the neutral profile obtained from the milk of a non-transgenic control animal, lane 3 is authentic 3-fucosyllactose, lane 4 is the neutral profile of a transgenic animal expressing FucT-IV, and lane 5 is the same material as in lane 4 but after specific fucosidase digestion.

Figure 16 illustrates FACE of selected fractions from control and transgenic milk oligosaccharide extracts after high pH anion exchange chromatography. Panel A represents a transgenic milk sample, and Panel B represents a non-transgenic control. Lane 1 of the gel represents dye, lane 2 represents transgenic oligosaccharide extract (fraction eluting at 19.5 minutes), lane 3 represents transgenic oligosaccharide extract (fraction elution at 20.0 minutes), lane 4 represents transgenic oligosaccharide extract (fraction eluting at 20.5 minutes), lane 5 represents authentic 3-fucosyllactose, lane 6 represents control oligosaccharide extract (fraction eluting at 20.0 minutes), and lane 7 represents control oligosaccharide

extract (fraction eluting at 20.5 minutes) and lane 8 represents the oligo ladder standard.

Figures 17 and 18 relate to the simultaneous transgenic expression of glycosyltransferases in mice.

5 Figure 17 is a chromatogram of the milk oligosaccharide profile obtained from a control, non-transgenic mammal.

Figure 18 is a chromatogram of milk obtained from a transgenic mammal expressing the human Fuc α 1-2-transferase and the murine Gal α 1-3 transferase.

10 Figures 19-21 relate to a method of transgenically expressing a bacterial glycosyltransferase in lactating mammary glands of mice.

Figure 19 shows the chromatogram obtained from the enzyme activity assay of a control L-cell plate.

15 Figures 20 and 21 show the oligosaccharide synthesized by transfected L cells expressing the bacterial N-acetylglucosaminyltransferase.

DETAILED DESCRIPTION OF THE INVENTION

20 As noted above, the subject invention relates to a method of producing one or more oligosaccharides and/or glycoconjugates in the milk of non-human transgenic mammals by the use of transgenic technology. One of the oligosaccharides which may be produced by the method of the invention (i.e., galactose α 1-3

25 galactose β 1-4 glucose) has a novel structure and has never been reported to exist as a free oligosaccharide. Such transgenically produced oligosaccharides and/or glycoconjugates, produced according to the present invention, may be added to infant formulas or to medical nutritionals given to the elderly

30 or to immunocompromised patients. The presently described method and variants thereof have the benefit of allowing scientists to custom design milk oligosaccharide profiles of transgenic animals.

Briefly, in order to produce the desired milk oligosaccharide or oligosaccharides, a segment of DNA encoding a particular enzyme of interest is isolated from a particular eukaryotic or prokaryotic species. This enzyme is responsible for the production of the secondary gene product or milk oligosaccharide. Subsequent to isolation, the DNA segment is inserted into a vector containing various genetic components such as, for example, a polyadenylation signal and a promoter. A portion of the vector containing the required components is then inserted into the pronucleus of an embryo of the same or of a different mammalian species from which the DNA segment was isolated. The embryo is then implanted into a recipient female, and birth is allowed to occur. Milk samples from the offspring contain the oligosaccharide or oligosaccharides of interest. Subsequent generations are also monitored for production of the oligosaccharide or oligosaccharides and thus incorporation of the gene or genes encoding the enzyme or enzymes of interest into their genomes. It should be stressed that more than one transgene may be introduced into the embryo thereby leading to production of more than one oligosaccharide. Thus, the potential of the present invention is endless. Each step of the present method is described in detail below.

The Enzyme

The enzyme of interest, which is ultimately expressed, is chosen based upon the oligosaccharide or oligosaccharides one wishes to produce in the milk of the non-human transgenic mammal. The enzyme may be, for example, a glycosyltransferase. A suitable glycosyltransferase includes, for example, a fucosyltransferase such as α -1,2-fucosyltransferase, fucosyltransferase IV or "H" fucose α -1,2-transferase, a galactosyltransferase such as gal α -1-3 transferase, an acetylase, a glucoronyltransferase, a glucosylepimerase, a sialyltransferase, a mannosyltransferase, a sulfotransferase, a β -acetylgalactosaminyltransferase and a N-acetylglucosaminyltransferase. The enzyme of choice is preferably a

galactosyltransferase, and more preferably UDP-Gal B-D- α 1,3 galactosyltransferase, a sialyltransferase, or a α 1-3/4 fucosyltransferase such a FucT-III or FucT-IV.

5 The glycosyltransferases encoded by the transgenes are targeted by signal sequences, either naturally encoded by cDNA or genomic DNA or which are added using standard genetic engineering techniques. They may be present in the lactogenic cells of the transgenic animals and may be either anchored to
10 intracellular membrane compartments (e.g., endoplasmic reticulum, golgi apparatus or secretory vesicles) of lactogenic cells or may exist as free catalytic polypeptides in the lumen of such compartments. Through appropriate intracellular
15 localization, the enzymes have access to sugar nucleotides or other sugar donors synthesized naturally by the lactating mammary gland cells or may obtain access to such cells from the bloodstream of the animal.

These donors are preferably sugar nucleotides.

20 The glycosyltransferases have the ability to catalyze the transfer of monosaccharide units from the sugar donors to acceptors such as lactose, other oligosaccharides, glycolipids or glycoproteins. The glycosyltransferases may have limited or
25 ample specificity for both donors and acceptors and may act by transferring more than one monosaccharide unit to a nascent or elongating glycoconjugate. Thus, larger oligosaccharides and/or polysaccharides can be synthesized by the reiterative or
iterative action of the glycosyltransferases. The glycosyltransferases may themselves be glycoproteins or non-
30 glycosylated glycoproteins or may or may not have additional co- and post-translational modifications.

It is important to note, again, that an oligosaccharide profile of a transgenic mammal can be designed based upon choice
35 of one or more appropriate enzymes which result in production of the oligosaccharides and glycoproteins of choice.

Furthermore, it should also be noted that the present invention allows for simultaneous expression of two or more enzymes and thus corresponding oligosaccharides and glycoproteins. Thus, potentially, a non-human transgenic mammal, for example, a cow, could be created which had the ability to produce many oligosaccharides present in human breast milk. The oligosaccharides could then be added to infant formula, for example, thereby providing the formula fed infant with the oligosaccharide-derived benefits received by the breast fed infant. Mammals, such as the cow described above, could then be allowed to mate (i.e., cross-bred) in order to obtain offspring which have the ability to produce a wide variety of valuable oligosaccharides in their milk.

15

Additionally, the present invention also encompasses the production of enzymes (e.g., glycosyltransferases) from invertebrates. Such invertebrates include, for example, Schistosoma mansoni or bacteria, such as from the Neisseriae genus. These enzymes may be expressed in active form in mammalian tissues during lactation.

20

The Non-Human Transgenic Mammal

A mammal, from which the cDNA encoding the enzyme of interest may be isolated is selected from the group consisting of, for example, a mouse, a rat, a rabbit, a pig, a goat, a sheep, a horse and a cow. However, any mammal may be utilized provided it has the ability to incorporate DNA encoding the enzyme of interest into its genome.

30

The embryo into which the cDNA is inserted may or may not correspond to the same species from which the DNA was initially isolated, depending upon whether heterologous expression or homologous expression of the enzyme is desired. More specifically, if one desires, for example, to produce a human enzyme which, in turn, leads to the production of a human oligosaccharide in the milk of a non-human, transgenic mammal

35

(i.e., heterologous expression of the enzyme), the transgenic mammal utilized is a non-human mammal such as, for example, a pig or a goat. However, if one wishes to produce, for example, a goat enzyme which, in turn, leads to the production of at least one oligosaccharide in the milk of a goat (i.e., homologous expression of the enzyme), the transgenic mammal utilized will be a goat.

With regard to homologous expression, the present invention allows one to synthesize oligosaccharides that are not present in nature such as, for example, galactose α 1-3 galactose β 1-4 glucose, as well as all naturally occurring oligosaccharides. Furthermore, the present invention allows for analysis of the synthesis efficiency of the enzyme encoded by the gene of interest. Also, since the enzyme is "native" to the mammal (i.e., derived from the same species), the potential of a self-immune reaction to the enzyme or intolerance in milk-fed progeny are reduced. Additionally, since some enzymes (e.g., glycosyltransferases) are found in some species and not others, the spectrum of potential oligosaccharides which may be transgenically synthesized in milk is increased by using available genes encoding homologous enzymes.

Also, with respect to homologous expression, it should be noted that homologous glycosyltransferase, for example, may be obtained and cloned from tissues other than the lactating mammary gland. For example, a murine glycosyltransferase which is normally expressed in liver and in other tissues, but is not expressed in lactating mammary glands of mice, may be expressed during lactation through the action of a lactogenically responsive promoter. The secondary gene products (i.e., oligosaccharides and glycoconjugates) will be present in the milk of the transgenic mice. Thus, as evidenced in Example I below, it is possible to synthesize oligosaccharides that are not normally synthesized by the lactating mammary gland of a given mammal as long as a glycosyltransferase, which is normally

expressed in other tissues, is transgenically expressed in the lactating mammary gland.

The Vector

5 The entity into which the cDNA (encoding the enzyme) is initially inserted is referred to a vector. The vector may be, for example, a bacterial plasmid (e.g., pBR-322). If a bacterial plasmid is used, it should contain at least a recombinant DNA sequence which directs gene expression and
10 protein synthesis, preferably during lactation, either a cDNA, gene or gene fragment encoding at least one glycosyltransferase, and a DNA sequence encoding a polyadenylation addition site required for proper messenger RNA processing. Alternatively, the vector may be constructed using other plasmids, cosmids,
15 phage DNA, viruses and other vectors specific for yeast, plant, mammalian and other suitable hosts.

 The promoter, which regulates expression of the cDNA, gene or gene fragment encoding the enzyme and which is operably
20 linked thereto, may be, for example, lactalbumin, casein, whey acidic protein (WAP) or another lactogenic promoter.

 The polyadenylation signal may be, for example, a mammalian or viral signal including, for instance, the poly-a signal of
25 SV-40T-antigen, ovalbumin or bovine growth hormone (bGHpdya).

Oligosaccharides

 Oligosaccharides which may be produced according to the method of the present invention include, for example, galactose
30 α 1-3galactose β 1-4 glucose, 2'fucosyllactose, 3'fucosyllactose, lacto-N-neo-tetraose, lacto-N-tetraose, for example, α -lacto-N-tetraose, lacto-N-fucopentaose I and its sialylated derivatives, lacto-N-fucopentaose II and its sialylated derivatives, lacto-N-fucopentaose III and its sialylated derivatives, lacto-N-
35 fucopentaose IV and its sialylated derivatives, lacto-N-fucopentaose V and its sialylated derivatives, lacto-N-di-

fucopentaose I and its sialylated derivatives, lacto-N-di-fucopentaose II and its sialylated derivatives, lacto-N-hexaose and its fucosylated derivatives, sialyltetrasaccharide a, b, and c and their fucosylated derivatives, other human milk and non-
5 human milk oligosaccharides.

Galactose α 1-3 galactose β 1-4 glucose and α 1-2-fucosyl-lactose are produced in the examples. The former compound represents the first novel, undescribed secondary gene product
10 synthesized by transgenic technology. In particular, galactose α 1-3 galactose β 1-4 glucose has never before been shown to exist in nature as a free oligosaccharide until the present invention. It is thought that this oligosaccharide may be used, for example, as an agent to induce tolerance to Gal α 1-3 antigen in
15 humans prior to xenotransplantation of non-human animal organs suchs as heart, liver and kidney.

It must be noted that the present invention encompasses the milk produced by the non-human transgenic mammal which contains
20 one or more of the above oligosaccharides. Additionally, however, the present invention also includes fractions of the milk as well as the oligosaccharides and glycoconjugates (e.g., glycoproteins) isolated or purified from the milk. Such fractions may include, for example, solids of the milk,
25 delipilidated or skim milk, milk from which the cream or lipids have been removed, the soluble carbohydrate fraction (i.e., specific oligosaccharides either alone or in combination with the mammal's naturally produced carbohydrates) and/or specific glycoconjugates or groups of glycoconjugates. Such fractions
30 may be obtained by methods known to those of ordinary skill in the art, for example, evaporation, lyophilization, crystallization, ultrafiltration, dialysis, or chromatography, (e.g., affinity, anion exchange or gel exclusion).

35 Additionally, the present invention includes glycoconjugates (e.g., human milk proteins and human serum

proteins) which are modified as a result of the action of the transgenically expressed enzymes. Such glycoconjugates may be endogenous or transgenically expressed. Examples of human milk proteins (i.e., glycoproteins) which may undergo modification
5 include secretory immunoglobulins, lysozyme, lactoferrin, kappa-casein, alpha-lactalbumin, beta-lactalbumin, lactoperoxidase and bile salt stimulated lipase. The enzyme may, for example, add a linkage to the glycoprotein which would not be present naturally in the glycoprotein (see Prieto et al., Journal of
10 Biological Chemistry 270:29515-29519 (1995)). Thus, the glycoprotein acts as a substrate for the enzyme.

It should be noted that either homologous or heterologous glycoproteins can be expressed transgenically in mammals which
15 are already expressing transgenes encoding glycosyltransferases. Thus, direct glycosylation may result, for example, from the action of endogenous as well as the transgenically expressed glycosyltransferases.

20 The present invention may be illustrated by the use of the following non-limiting examples:

The following reagents and equipment were required for use in Example I:

25

REAGENTS:

- 1) 500mM sodium hydroxide and 200mM sodium hydroxide, RICCA Chemical Company (Arlington, TX), carbonate free, National
Institute of Standards and Technology (NIST)-traceable (Baxter
30 Inc., McGaw Park, IL);
- 2) Sodium acetate, ACS grade (Sigma, St. Louis, MO);
- 3) FACE reagent kit (Glyko Inc., Novato, CA);
- 4) Milli-Q water (Millipore, Bedford, MA); and
- 5) Sulfuric Acid Ultrex Ultrapure Grade (Baxter).

35

EQUIPMENT:

Sample Drying: All samples and standards were dried using a

Speed Vac Plus SC210A equipped with a Refrigerated Vapor Trap RVT4104 (Savant, Farmingdale, IL).

Example I

5 Expression of Murine UDP-Gal β -D- α 1,3 Galactosyltransferase (Homologous Expression)

A. Preparation of Plasmid and Creation of Transgenic Mouse Embryos:

10 The genetic construct was prepared at the Edison Biotechnology Institute at Ohio University (Athens, Ohio). A cDNA encoding the murine UDP-Gal: β -D-Gal α 1,3 galactosyltransferase was inserted into a plasmid containing the polyadenylation signal of bovine growth hormone (bGHpolyA) and
15 the lactogenically-responsive murine whey acidic protein (WAP) promoter (see Figure 1). An EcoRI-BamHI fragment was microinjected into the pronuclei of mouse embryos. The presence of the gene was detected by tail slot blots. Milk from lactating females was collected and stored frozen at -70°C until
20 shipped to Ross Labs for carbohydrate analysis.

B. High Performance Anion Exchange Chromatography Analysis of Oligosaccharides of A:

25 Mouse milk oligosaccharide standards were analyzed using a Dionex Bio-LC System, equipped with the following:

Pump:	Dionex Advanced Gradient Pump
Detector:	Dionex Pulsed Electrochemical Detector with gold working electrode and pH/reference electrode
30 Autosampler:	Spectrophysics Refrigerated AS3500
Columns:	Method 1: 1 Dionex CarboPac PA1 analytical column (4 X 250 mm) preceded by a guard (4 X 50 mm)

35

Method 2: 2 Dionex CarboPac PA1

analytical columns (4 X 250mm each,
connected in serial) preceded by a
guard (4 X 50 mm)

5 Chemical Suppression:

Dionex AMMS-II Anion Micromembrane
Suppression, (installed after detector
but before fraction collector, used to
neutralize samples), regenerated by
10 0.15% sulfuric acid using Dionex
AutoRegen Apparatus and Anion
Regenerate Cartridge

Fraction Collector:

BioRad Model 2110 (BioRad Inc.,
15 Hercules, CA)

All equipment was purchased from Dionex, Inc. (Sunnyvale, CA)
unless otherwise noted. Eluants used were a combination of (A)
Milli-Q water (or equivalent), (B) 500 mM sodium hydroxide, (C)
600mM sodium acetate in 100mM sodium hydroxide, and/or (D) 200
20 mM sodium hydroxide, at a flow rate of 1.0 ml/min. Table I
below contains the eluant profiles for the two methods used.
Detector settings are as listed in Table II below. Injection
volume was 20µl.

25 Dionex Analysis of Samples and Standard: All samples, and/or
standards were analyzed as prepared without further dilution.

WO 99/11773

PCT/US98/18383

20

TABLE I: Eluant Profiles

Method 1:

Time (min)	% A	% B	% C	% D
0.0	50	0	0	50
12.0	50	0	0	50
12.1	46	0	7	47
20.0	46	0	7	47
20.1	45	0	10	45
27.0	45	0	10	45
27.1	25	0	50	25
32.0	25	0	50	25
32.1	50	0	0	50
59.0	50	0	0	50

Method 2:

Time (min)	% A	% B	% C	% D
0.0	99	1	0	0
60.0	0	100	0	0
60.1	99	1	0	0
75.0	99	1	0	0

A=Milli-Q Water

B=500 mM NaOH

C= 600 mM NaOHAc/100 mM NaOH

D=200 mM NaOH

TABLE II: Detector Settings

Integration Parameters:	Method 1:	Method 2:
Starting Peak Width	50	8.0
Peak Threshold	0.5	25
Peak Area Reject	500	1000
PED Recorder Range	0.100 uC	0.300 uC

Method 1:

Waveform:		Integration:	
Time (Sec)	Poten-tial (V)	Begin (Sec)	End (Sec)
0.00	0.40	0.30	0.50
0.50	0.40		
0.51	0.90		
0.59	0.90		
0.60	-0.30		
0.65	-0.30		

Method 2:

Waveform:		Integration:	
Time (Sec)	Poten-tial (V)	Begin (Sec)	End (Sec)
0.00	0.00	0.20	0.40
0.40	0.05		
0.41	0.75		
0.60	0.75		
0.61	-0.15		
1.00	-0.15		

C. Fluophore Assisted Carbohydrate Electrophoresis (FACE): Samples and standards were prepared for FACE using reagent kit (Glyko) and directions supplied by manufacturer.

5 Suggested electrophoresis conditions were followed. Gels were imaged using a FACE Imager (Glyko).

A standard of synthetic Gal α 1-3Gal β 1-4Glc, 15 mMole (7.5 μ g), was resuspended in 37.5 μ l water to make a 200 ppm solution. 4 μ l of this solution were diluted with 36 μ l of
 10 water and analyzed by HPAEC (see Figure 2 using Method 1). (The remaining stock standard was separated into 5 μ l aliquotes and stored frozen for future analyses.) Fractions (0.5ml) were collected, and the fractions corresponding to standard peak (38-44) were pooled and dried overnight in Speed Vac at room
 15 temperature. The resulting dried pool was labeled for 3 hours at 45°C per manufacturer's instructions and dried. The sample was then resuspended in 14 μ l of water, and 2 μ l (105 pmol) were diluted in 2 μ l of loading buffer and loaded onto a polyacrylamide gel. The gel was electrophoresed per
 20 manufacturer's instructions. A fluorographic image of the gel is shown in Figure 3.

As noted in the Brief Description of the Drawings section, Figure 5 shows an example of a negative transgenic, positive transgenic, and negative control mouse milk oligosaccharide
 25 profile as analyze using HPAEC Method 2. The large peak eluting

at 31-32 minutes is presumed to be the trisaccharide. HPAEC Method 1 generated a small artifact peak that eluted very close to the trisacchare standard and remained to run using Method 2, so to verify that the "new" peak detected was indeed the trisaccharide, the oligosaccharide extracts from these samples were labeled and tested by FACE. 8 µl of the oligosaccharide extracts were dried and labeled according to kit instructions. The labeled samples were resuspended in 10 µl of water, and 2 µl were loaded onto the gel. A previously labeled frozen sample of the trisaccharide standard was also run. The image of this gel is found in Figure 6. The band that runs concurrent with the G3 starch by hydrolysate standard band is the trisaccharide.

D. Extraction of Mouse Milk:

Mouse milk samples, which had been stored at -70°C, were thawed at room temperature. 100µL were pipetted into a 500µL Eppendorf centrifuge tube, and were centrifuged for 20 minutes at 11000 rpm using a BioFuge 15 (Baxter, McGaw Park, IL). The lipid pad was carefully removed, and the middle layer was transferred to a second 500µL tube. A two-fold volume of cold (4°C) ethanol was added, and the vial was vortexed and placed on ice for at least twenty minutes. The vial was then centrifuged as before. The clear supernatant was transferred to a third vial, and this extract was dried at medium heat overnight in a Speed Vac. The protein pellets were also dried, and were stored at -70°C until analysis. The dried oligosaccharide extracts were resuspended in 100µL water, and stored at -70°C until analysis. Due to low quantity of available standard, it was not put through this entire procedure; however, other oligosaccharides have undergone this extraction method with no substantial losses in quantity (data not shown).

Table III displays a summary of all WAP/GT transgenic milk tested, sorted by the Founder numbers. Of the 23 "transgenic" samples tested (where "transgenic" is used to define a mouse that has tested positive for copies of the transgene in tail slot blots), 10 animals, or 44% of all animals in relevant germ lines, were positive for the synthesis of the trisachharide.

WO 99/11773

PCT/US98/18383

23

(Note: the samples identified as "Founder B6SJL" were the negative control/non-transgenic mice.)

TABLE III

5

19	B6SJL						
22	B6SJL						
21	B6SJL						
25	B6SJL						
4	8	1	19	hemi	3061.187	negative	3
5	8	1	20	hemi	3061.187	negative	
3	8	1	22	hemi	3061.187	negative	
6	8	2	13	homo	3147.19	negative	
11	8	2	23	homo	3061.242	negative	
1	64	0			3061.11	negative	?
13	82	1	71	hemi	3147.23	negative	1-2
7	98	0			3147.2	negative	1
16	106	1	93	hemi	3147.199	negative	1
29	106	2	61	hemi	3147.200	negative	
8	109	1	48	hemi	3147.21	positive	1-2
15	109	1	67	hemi	3147.24	positive	
20	109	1	118	hemi	3147.2	negative	
22	109	2	50	hemi	3147.2	negative	
27	109	2	51	hemi	3147.2	positive	
17	109	2	51	hemi	3147.2	positive	
18	109	2	53	hemi	3147.2	positive	
23	109	2	54	hemi	3147.2	positive	
2	156	0			3061.187	positive	5
9	156	0			3061.242	positive	
10	156	1	42	hemi	3061.242	positive	
23	156	1	100	hemi	3147.199	negative	
26	156	2	27	hemi	3147.210	positive	
14	193	0				not tested	1
24	193	1	120	hemi		not tested	

Note in Table III that milk samples from the same founder may or may not test positive for the synthesis of the trisaccharide; possession of a copy (or copies) of the transgene in the DNA does not guarantee that the mouse will be able to synthesize the trisaccharide. (For example, see results of milks from various animals related to Founder 109.) It may be possible that these samples contained the trisaccharide; however, it was produced below the detection limit of the method (approx. 10 ppm).

E. Spiking of Control Mouse Milk with Trisaccharide Standard:

1852 pmol of trisaccharide standard were dried in a 500 μ l eppendorf centrifuge tube. 100 μ l control (non-transgenic) mouse milk was added, and the vial contents were mixed well. This spiked milk sample was then defatted and deproteinized as described above. 45 μ l ethanol extract was recovered, of which 20 μ l (containing a theoretical 820 pmol of trisaccharide) were dried and labeled 3 hours at 45°C for oligosaccharide analysis (Glyko). After drying, the sample was resuspended in 10 μ l water, and 2 μ l were loaded onto a FACE gel. The image is found in Figure 4. An unspiked control milk sample was also analyzed for comparison.

All samples and/or standards in C and D were analyzed using HPAEC as prepared, without further dilution.

Example II

Expression of Human α 1,2-Fucosyltransferase in Rabbits (Heterologous Expression)

Creation of transgenic embryos was carried out in collaboration with Dr. John Kopchick and his colleagues at the Edison Biotechnology Institute at Ohio University in Athens, Ohio, and with Zdzislaw Smorag at the Zootechnics Institute in

Balice, Poland. 90% of the rabbits used were New Zealand bred, with the remaining 10% being California bred.

A. Preparation of Transgenic Zygotes:

5 Zygotes were collected from superovulated female rabbits which were 4-6 months old. Superovulation was achieved by standard procedures employing 50-100 i.u. of Pregnant Mare Serum Gonadotropin (PMSG) injected intramuscularly, followed by 50-100 i.u. of Human Chorionic Gonadotropin (HCG) injected
10 intravenously 72 hours after PMSG. Immediately after HCG injection, superovulated females were mated or inseminated with fresh or frozen semen from 5 month old males. 20-22 hours after insemination or mating, females were slaughtered, and zygotes were collected by oviduct flushing supplemented with PBS.
15 Zygotes were microinjected with DNA (i.e., a linear DNA fragment excised from a previously described plasmid (Prieto et al., Journal of Biological Chemistry 270:29515-29519 (1995)) containing a transcriptional regulatory region which included the murine whey acidic protein promoter, a cDNA encoding the
20 human H- α 1-2 fucosyltransferase and a sequence encoding the polyadenylation signal of bovine growth hormone) encoding 2'-fucosyltransferase at 2-6 ng/ml, 1-2 hours after flushing, using an inverted microscope with Namarski optics, and micropipets connected with Da Venbrine or Leitz micromanipulators.

25

B. Implantation of Transgenic Zygotes & Collection of Milk:

One hour after microinjection, the zygotes were surgically transferred into oviducts of synchronized, pseudopregnant
30 recipients who were 5-6 months old. The progress report of the insemination project is given in Table IV. The resulting pups were analyzed for successful transcription of the DNA by dot blot analyses. Milk from two of these animals, along with that from non-transgenic controls, was collected and analyzed.

Table IV: Insemination Progress Report

Group Number	No. of Inseminated Females	No. of Pregnant Females	No. of Rabbits Born Alive	No. of Stillborn Rabbits
I	8	7	26	1
II	4	4	16	8
III	20	16	67	17
IV	20	7	**	**
Total	52	34	109	26

(**Information not available at this time)

5 C. Preparation of Milk Obtained From Pups:

Milk samples from transgenic and control rabbits were diluted 1:3 with ethanol and shipped to Ross Laboratories (Columbus, OH). They were vortexed well and centrifuged 10 minutes at 10,000 rpm. The ethanol layer was removed and placed
10 in a clean vial for oligosaccharide analysis, and the precipitates were frozen for future protein analysis.

D. Oligosaccharide Profiling:

Fifty µl of each of the ethanol extracts were diluted with
15 50 µl water. The dilutions were filtered through pre-rinsed 30,000 MWCO filters (Microcon, Amicon, Inc., Beverly, MA), and were analyzed using HPAEC Method 1 (see Appendix I at end of this example). Spiked samples of one of each control and transgenic samples were prepared by diluting a 50 µl sample with
20 48 µl water and 2 µl 1000 ppm 2'-fucosyllactose, and filtering as before. These samples were also analyzed. A second set of milk samples was analyzed as received, with no further dilution.

Of the remaining ethanol extracts, 240 µl were dried in a Speed Vac (Savant, Inc., Farmingdale, IL). The extracts were
25 then resuspended to volume with 7% IPA and filtered through

Microcon 30,000 MWCO as before. 20 μ l of each sample from set one, and 50 μ l from each sample of set two, were dried and labeled overnight according to manufacture's instructions (Glyko, Inc., Novato, CA). They were then labeled overnight according to manufacturer's instruction (Glyko, Inc.). The labeled samples were resuspended in 10 μ l water. 2 μ l of each were loaded onto an oligosaccharide gel and electrophoresed.

E. Fucosidase Treatment:

25 μ l of the filtered IPA dilutions from set one were dried in 2 separate vials, along with two 2'-fucosyllactose (2'-FL) aliquots of 1000 ppm. The samples were resuspended in 25 μ L 1x PBS, pH 8.5. 200 mU α 1,2-fucosidase from Arthrobacter oxidans (Takara-Panvera, Madison, WI) in PBS were added to each one of the vials. The same volume of a boiled (deactivated) enzyme was added to the second vial. The samples were incubated at 37 °C overnight under a toluene atmosphere. They were filtered through rinsed Centricon 10,000 MWCO filters (Amicon), using 950 μ l water to rinse. The filtrates were then passed through On-Guard A - strong anion-exchange cartridges (Dionex), which were prepared according to manufacturer's instructions. The samples were rinsed through with 200 μ L water. The collected filtrates were frozen, dried, and labeled overnight for FACE analysis (Glyko). The samples were diluted in 10 μ l water, and a 2 μ l aliquot of this material was subjected to electrophoresis and imaging.

F. SDS-PAGE and Western Blots of Glycoproteins:

The proteins pellets were thawed to room temperature, washed twice with cold 68% ethanol, and dried. They were resuspended in 2x SDS denaturing buffer. The samples were heated in boiling water for 5 minutes to denature the proteins and were cooled. Dilutions were made using 1x SDS buffer. Samples and Prestained Low Molecular Weight Standard (Sigma, St. Louis, MO) were loaded onto a polyacrylamide gel (Novex, Inc.,

San Diego, CA) and electrophoresed in the Xcell II Mini Cell System (also from Novex). The gel thus obtained was transferred to a PVDF membrane, and probed with *Ulex europaeus* Agglutinin I (UEA I) (peroxidase labeled, Sigma) overnight. After washing
5 with TBS-Tween 20, the gel was developed using a DAB-Hydrogen Peroxide Staining solution (Sigma). The dried gel was scanned and digitized using an ordinary 300 dpi computer scanner.

The results were as follows:

10 Figure 7 contains the chromatographic oligosaccharide profiles of control non-transgenic, positive transgenic, and spiked-positive transgenic rabbit milk samples as obtained from one sample. Transgenic sample #1 contained 2'-FL, a fact
15 corroborated by the spiked profile. Note the large difference in lactose concentration between the control and the transgenic sample. A second transgenic sample (#2), which was found to be negative (containing no 2'-FL), is not shown. Of the profiles found in Figure 8, samples #4 (panel C) and #5 (panel D)
20 contained 2'-FL in quantities detectable by this method. This sample exhibited a marked decrease in lactose concentration while sample #3 (panel B) had no detectable amounts of lactose.

To further verify the nature of the oligosaccharide present in milk samples from transgenic animals, oligosaccharides from
25 control non-transgenic and transgenic milk were subjected to hydrolysis by α 1-2 fucosidase. This enzyme exclusively hydrolyzes fucose 1-2 linkages. Results are shown in Figure 9. Authentic 2'-fucosyllactose (2'-FL) is shown in Lane 1 while untreated oligosaccharides from a transgenic sample are shown in
30 Lane 2. Lane 3 shows the same material as in Lane 2, but after fucosidase treatment. A band comigrating with authentic 2'-FL is clearly observable in Lane 2. This band disappeared after fucosidase treatment, further supporting the presence of 2'-FL in the transgenic milk sample. Oligosaccharides from a control
35 non-transgenic sample were substantially unchanged after

fucosidase treatment (see Lanes 4 and 5).

FACE oligosaccharide profiles of additional milk samples are shown in Figure 10. Lane 2 of this figure shows the resolved labeled oligosaccharides from milk of sample #5. Lane 3 shows the oligosaccharides of another transgenic milk sample. Lane 4 shows the oligosaccharides of sample #4. Lane 5 is the profile from a control non-transgenic animal, and Lane 7 is sample #3. These results corroborate the chromatographic profiles of Figure 8 and further support the concept that some transgenic milk samples were devoid of lactose.

Figure 11 compares Western blots of milk protein samples #1 and #2, probed with UEA-I. Positive bands are seen only in Lane 2, one of the positive transgenic samples. Neither sample #2 nor the non-transgenic control, contained proteins which are glycosylated with fucose in the α -1,2 linkage. Western blots for additional samples are shown in Figure 12. As expected, sample #4 contained proteins which are fucosylated in the alpha-1,2 position. Sample #3, however, also contained similarly decorated proteins. This result was not expected due to the lack of detectable 2'-FL which the other two methods missed. All other samples analyzed to date (including those from mice) have contained, at least, fucosylated proteins. The Coomassie stain of a gel identical to that found in Figure 12 is presented as Figure 13. This figure illustrates that, although no proteins were detected by UEA-I in the non-transgenic controls, there were many proteins present in the sample. As a contrast, compare the total proteins found in samples #3 and #4 to the bands detected in the UEA-I blot. Although they were present in almost non-detectable quantities, they were strongly detected by the lectin.

WO 99/11773

PCT/US98/18383

30

Appendix I: Chromatography Conditions

Oligosaccharide Analysis by High-Pressure Anion-Exchange Chromatography:

Samples were analyzed using a Dionex Bio-LC system, equipped with a
5 Pulsed-electrochemical detector, employing a single analytical CarboPac
PA-1 column with guard. Conditions were as follows:

Sensitivity: 0.200C

Run Time: 45 minutes

10 Peak Width: 8.0 seconds

Peak Threshold: 25.00

Peak Area Reject: 1000

Sample Volume: 20L

15 Gradient Program:

0 - 12 minutes:	100mM NaOH
12.1- 20 minutes:	42mM NaOAc in 100mM NaOH
20. 1- 27 minutes:	60mM NaOAc in 100mM NaOH
27.1- 32 minutes:	300mM NaOAc in 100mM NaOH
20 32.1 - 45 minutes:	1 00mM NaOH

PED Program:

Waveform:

Integration:

	<u>Time (sec):</u>	<u>Potential (V):</u>	<u>Begin (sec):</u>	<u>End (sec):</u>
25	0.00	0.05	0.20	0.40
	0.40	0.05		
	0.41	0.75		
	0.60	0.75		
	0.61	-0.15		
30	1 .00	-0.15		

Example IIIExpression of Human Fucosyltransferase IV in Mice
(Heterologous Expression)

5 A human fucosyltransferase (i.e., the "H" fucose α 1-2-transferase) has previously been expressed in lactating mammary glands of mice (Prieto et al., Journal of Biological Chemistry, 270:29515-19 (1995)). Its expression resulted in the production of oligosaccharides and glycoproteins synthesized by the enzyme.

10 In order to demonstrate the potential, broad application of this system for the production of secondary gene products, the present inventors decided to transgenically express a glycosyltransferase, other than the human H-fucose α 1-2-transferase, in mice. One such enzyme is fucosyltransferase IV

15 (Kukosawa-Latallo et al., Genes and Development 4:1288-1303 (1989)). This enzyme is present in certain samples of human milk (Serenella et al., Glycoconjugate Journal 6:101-114 (1989)) and is of particular interest because it is responsible for the synthesis of certain blood group antigens (Legault et al., The

20 Journal of Biological Chemistry 270:20987-20996 and Ginsburg et al., in Immunology of the Erythrocyte, Alan R. Liss, Inc., New York, New York). These blood group antigens are important because they are elevated during presence of cancer (Orntof et al., The Journal of Biological Chemistry 271:32260-32268 (1996))

25 and have been implicated in cell adhesion phenomenae such as inflammation (Lowe et al., The Journal of Biological Chemistry 266:17467-17477)). The expression of the human enzyme fucosyltransferase IV and synthesis of 3-fucosyllactose were carried out as described below:

30

The following reagents were utilized:

- 500mM sodium hydroxide and 200mM sodium hydroxide, RICCA Chemical Company (Arlington, TX), carbonate free
- NIST-traceable (Baxter Inc., McGaw Park, IL)
- 35 • sodium acetate, ACS grade, (Sigma, St. Louis, MO.)

- FACE Reagent Kit (Glyko Inc., Novato, CA)
- Milli-Q Water (Millipore, Bedford, MA)
- Sulfuric Acid Ultrex Ultrapure Grade (Baxter)

5 Sample Drying: All samples and standards were dried using a Speed Vac Plus SC210A equipped with a Refrigerated Vapor Trap RVT4104 (Savant, Farmingdale, IL).

A. Preparation of Plasmid and Creation of Transgenic Embryos:

10 The genetic construct was prepared at the Edison Biotechnology Institute at Ohio University (Athens, Ohio). A cDNA (i.e., a linear DNA fragment excised from a previous described plasmid (Prieto et al., Journal of Biological Chemistry 270:29515-29519 (1995)) containing a transcriptional regulatory region which included the whey acidic protein
15 promoter, a cDNA encoding the human α 1-3/4- fucosyltransferase (FucT-IV) and a sequence encoding the polyadenylation signal of bovine growth hormone) encoding the human fucosyltransferase (IV) was inserted into a plasmid containing the polyadenylation signal of bovine growth hormone (bGH polyA) and the
20 lactogenically-responsive murine whey acidic protein (WAP) promoter. An EcoRI-BamHI fragment was microinjected into the pronuclei of mouse embryos. The presence of the gene was detected by tail slot blots. Milk from lactating females was collected and stored frozen at -70°C until shipped to Ross
25 Laboratories (Columbus, OH) for carbohydrate analysis. Three transgenic females were obtained. Milk from two of these females was collected and analyzed.

B. HPAEC Analysis of Samples and Standards:

30 Mouse milk oligosaccharides and oligosaccharide standards were analyzed using a Dionex Bio-LC System, equipped with the following:

35	<u>Pump:</u>	Dionex Advanced Gradient Pump
	<u>Detector:</u>	Dionex Pulsed Electrochemical Detector

with gold working electrode and
pH/reference electrode

Autosampler:

Spectrophysics Refrigerated AS3500

Columns:

2 Dionex CarboPac PA1 analytical
columns (4 x 250 mm each, connected in
serial) preceded by a guard (4 x 50
mm)

Chemical Suppression: Dionex AMMS-II Anion Micromembrane

Suppression, (installed after detector
but before fraction collector, used to
neutralize samples), regenerated by
0.15% sulfuric acid using Dionex
AutoRegen Apparatus and Anion

Regenerant Cartridge

Fraction Collector: BioRad Model 2110 (BioRad Inc.,
Hercules, CA)

All equipment was purchased from Dionex, Inc. (Sunnyvale, CA)
unless otherwise noted. Eluants used were a combination of (A)
Milli-Q water (or equivalent), (B) 500 mM sodium hydroxide, (C)
600 mM sodium acetate in 100 mM sodium hydroxide, and/or (D) 200
mM sodium hydroxide, at a flow rate of 1.0 ml/min. Table V
below contains the eluant profiles for the two methods used.
Detector settings are as listed in Table VI below. Injection
volume was 20 µL.

All samples and/or standards were analyzed as prepared
without further dilution. When further characterizations was
deemed necessary. Fractions were collected (0.5 minutes per
fraction) and dried in their collection tubes. These samples
were then labeled for FACE analysis. Whenever possible,
chromatograms were obtained for transgenic and control animals.
As preliminary characterization step, aliquots were spiked with
authentic standards of 3-fucosyllactose. In this fashion,
coelution of neooligosaccharide with authentic 3-fucosyllactose
was demonstrated.

WO 99/11773

PCT/US98/18383

34

TABLE V : Eluant Profiles

A= Milli-Q Water

B = 500 mM NaOH

C = 600 mM NaOHAc/100 mM NaOH

D = 200mM NaOH

Method

Time (min)	0/0 A	0/0 B	0/0 C	0/0 D
0.0	99	1	0	0
60.0	0	100	0	0
60.1	99	1	0	0
75.0	99	1	0	0

TABLE VI: Detector Settings

Integration Parameters:	Method 1:	Method 2:
Starting Peak Width	50	8.0
Peak Threshold	0.5	25
Peak Area Reject	500	1000
PED Recorder Range	0.100 uC	0.300 uC

Method

Waveform:

Integration:

Time (sec)	Potential (v)	Begin (sec)	End (sec)
0.00	0.00	0.20	0.40
0.40	0.05		
0.41	0.75		
0.60	0.75		
0.61	-0.15		
1.00	-0.15		

5

C. Fluorophore Assisted Carbohydrate Electrophoresis (FACE):

10 Samples and standards were prepared for FACE using a reagent kit and directions supplied by manufacturer (Glyko, Novalto, CA). Suggested electrophoresis conditions were followed. Gels were imaged using a FACE Imager (Glyko).

15 Samples of milk oligosaccharide extracts (10 μ l) were dried and labeled 3 hours at 45°C for oligosaccharide analysis (Glyko) as previously described. After drying, the sample was resuspended in 10 μ l of water, and 2 μ l were loaded onto a polyacrylamide oligosaccharide resolving gel. Images of the electrophoretograms were obtained using a Glyko Face Imager.

20 D. Extraction of Mouse Milk:

Mouse milk samples which had been stored at -70°C, were thawed at room temperature and mixed by vortexing. 50 μ l were pipetted into a 500 μ l Eppendorf and 100 μ l of cold absolute ethanol were added. The resulting mixture was vortexed and

centrifuged for 15 minutes at 4°C and 11000 rpm using a BioFuge 15 (Baxter, McGaw Park, IL). After centrifugation, 85 µl of clear supernatant were transferred into another 500 µl centrifuge tube, and 199 µl of distilled deionized water were added. The resulting mixture was mixed well, and 50 µl were transferred to an autosampler vial, diluted 1:1 with water and submitted to High pH Anion Exchange Chromatography (HPAEC). This final solution was considered the oligosaccharide extract of the milk sample.

10

E. Spiking of Milk with Authentic 3-Fucosyllactose:

A solution of 3-fucosyllactose Galβ1-4[Fucα1-3]Glc of 50 mg/liter was added to 50 µl of the oligosaccharide extract described above and mixed well. This spiked milk sample was then directly submitted to chromatography and compared with an oligosaccharide extract that was diluted with a volume of water.

15

F. Exoglycosidase Treatments:

Fucosidases from Corynebacterium sp. (α1-2 specific) and Streptomyces sp. (α1-3/4 specific) were from Takara Panvera, (Madison, WI). E. coli and other chemicals used were of the highest purity available and, unless otherwise indicated, were from Sigma Chemical Co. (St. Louis, MO).

20

G. Separation of Neutral and Charged Oligosaccharides:

Oligosaccharide extracts (200 µl aliquots) were separated into neutral and charged fractions to facilitate detection and identification of neooligosaccharides synthesized by the action of the transgene encoded fucosyltransferase.

25

A column (3 ml) of DE-52 (diethylaminoethyl cellulose, Whatman, Maidstone, England) was equilibrated in 0.002 M pyridine acetate buffer pH 5.4 by passing approximately 20 volumes of the buffer through it. The oligosaccharide extract

aliquot was applied to the column, allowed to permeate into the resin and to rest for 5 to 10 minutes. The neutral oligosaccharides were then eluted by eluting the column with 6 volumes of the same buffer. The fraction thus obtained was
5 dried and prepared for labeling and fluorophore assisted carbohydrate electrophoresis. The charged fraction was subsequently eluted from the column by rinsing it with five volumes of 0.2 M pyridine acetate buffer.

10 The results of the above procedures were as follows:

Figure 14 shows chromatograms for control and transgenic milk oligosaccharide extracts (Panels A and B, respectively). It can be clearly observed that a peak signal was generated by
15 milk obtained from an animal transfected with the fucosyltransferase FucT-IV, while there was only a trace in the non-transgenic control animal milk. Additionally, material shown in Panel B was spiked with authentic 3-fucosyllactose to determine if the neooligosaccharide present in milk of the
20 transgenic animal was indeed 3-fucosyllactose (Panel C). The elution of this carbohydrate is further consistent with the independent elution of authentic 3-fucosyllactose indicated by the arrow in Panel B.

25 Additionally, oligosaccharide extracts from control and transgenic animals were separated into neutral oligosaccharides and sialyoligosaccharides or negatively charged structures. This allowed for simpler images when labeled oligosaccharides where resolved by gel electrophoresis. Figure 15 is the image
30 of a gel used to resolve neutral labeled oligosaccharides. (Lane 1 is a labeled starch hydrolyzate used as a molecular weight standard, lane two is the neutral profile obtained from the milk of a non-transgenic control animal, lane 3 is authentic 3 fucosyllactose, lane 4 is the neutral profile of a transgenic
35 animal expressing FucT-IV and lane 5 is the same material as in

lane 4 but after specific fucosidase digestion.) A band which comigrated with authentic 3 fucosyllactose was only clearly visible in the profile from the transgenic sample, and the band disappeared when treated with specific 3-fucosidase.

5 Additionally, the material was not susceptible to hydrolysis by α 1-2-fucosidase.

To further characterize the neooligosaccharide, fractions were collected directly from the chromatography eluants.

10 Oligosaccharide extracts were subjected to HPAEC as described above and eluants were collected in fractions. Each fraction corresponded to 0.5 minutes of elution. The collected fractions are indicated by brackets in Figure 16 Panel A for a transgenic milk sample and Panel B for a non-transgenic control. These
15 fractions were labeled and applied to FACE as indicated in the lane identification legend of the electrophoretogram image in Figure 16.

Both HPAEC and FACE show that the non-transgenic animal had
20 only traces of an oligosaccharide comigrating with authentic 3 fucosyllactose (Lane 5). However, a fraction from the oligosaccharide extract from the milk sample obtained from a transgenic animal generated a clear signal which comigrated with 3-fucosyllactose. This further supports the concept that
25 3-fucosyllactose is synthesized by the expression of the transgene.

Example IV

30 Simultaneous Expression of Two Transgenic Fucosyltransferases Through Interbreeding of Two Previously Established Murine Transgenic Lines

A female mouse from a stable transgenic line expressing the human fucosyltransferase "H", driven by a lactogenic promoter
35 (Prieto et al., Journal of Biological Chemistry, 270:29515-29519

(1995)) was allowed to mate with a male mouse from a stable transgenic line expressing the murine Gal α 1-3 transferase, also driven by a lactogenic promoter (see Example I).

5 The resulting progeny was assessed for the presence of the transgenes, in their genome, utilizing tail biopsies and probing with suitable hybridizing probes. Females deemed to be positive for both transgenes were further mated and pregnancy was allowed to proceed to term. Milk was obtained from one of these
10 females, and the carbohydrate profile was determined by High pH Anion Exchange Chromatography (HPAEC), as described in Examples I and II.

As observed in Figures 17 and 18, a major signal can be
15 observed at the elution time of 2'-fucosyllactose while a minor signal can be observed precisely at the elution time of the synthetic trisaccharide Gal α 1-3 Gal β 1-4Glc. Elution positions of authentic standards are indicated.

20 In particular, the above results indicate that in order to obtain transgenic animals which produce milk containing complex oligosaccharide and glycoprotein profiles, it is not necessary to start *de novo* and subsequently inject mice embryos with new glycosyltransferase encoding constructs. Interbreeding of
25 transgenic animals expressing different glycosyltransferases will result in animals that inherit transferases from their parents according to Mendelian genetics. This, in turn, will result in the synthesis of the relevant secondary gene products.

30 Example V

Transgenic Expression of a Bacterial Glycosyltransferase (i.e., UDP-GlcNAc:Gal β 1-4) in Lactating Mammary Glands of Mice

A GlcNAc-transferase from Neisseria polysaccharea was used.
35 The codon bias of the gene encoding the enzyme was altered to

favor the use of codons commonly found in mammalian genes. The "rebiased" gene was used to transfect murine "L" cells commonly used as murine culture cell lines. Once activity is proven in these mammalian cells, the resulting DNA encoding the GlcNAc-
5 transferase was used to prepare a construct amenable for transgenic expression during lactogenesis in mice.

GlcNAc-transferase activity was assessed by a liquid phase synthesis assay. Mouse "L" cells (i.e., a pellet from a petri
10 dish) are resuspended in Phosphate Buffered Saline buffer containing 1mM CaCl₂, 20 mM lactose and 20 mM UDP-GlcNAc. The resulting mixture was sonicated for 5 minutes and incubated overnight at 37 degrees centigrade. The resulting incubation mixture was then filtered through microcon (Amicon, Beverly, MA)
15 centricon (10,000 AMU, MW cutoff) filters, and the filtrate was diluted for HPAEC analysis.

Oligosaccharide extracts were prepared as reported in Example I. Figures 19-21 clearly indicate that the bacterial
20 enzyme was successfully expressed in mammalian tissues.

The milk of the transgenic animals may contain the tetrasaccharide lacto-N-neotetraose (NnnT) as a result of the expression of the N-acetyl-glucosaminyl-transferase.
25

CLAIMS

1. A method for producing a non-human, transgenic mammal whose somatic and germ cells contain at least one transgene, wherein expression of said at least one transgene results in the production of oligosaccharides and glycoproteins in the milk of said mammal, the method comprising the steps of:

(a) preparing at least one transgene, said at least one transgene comprising in operable association 1) at least one expression regulatory sequence functional in mammary secretory cells, 2) a DNA sequence encoding a signal sequence functional in mammary secretory cells, and 3) a DNA sequence encoding an enzyme;

(b) introducing said at least one transgene into a non-human, mammalian embryo, and transferring the resulting embryo into a recipient female;

(c) identifying at least one female offspring, where expression of said at least one transgene results in the production of at least one enzyme which then catalyzes the production of oligosaccharides and glycoproteins in the milk of said mammal.

2. The method of claim 1, wherein said non-human, transgenic mammal is selected from the group consisting of a mouse, a rat, a rabbit, a pig, a goat, a sheep and a cow.

3. The method of claim 1, wherein said at least one enzyme is a glycosyltransferase.

4. The method of claim 3, wherein said glycosyltransferase is selected from the group consisting of a fucosyltransferase, a galactosyltransferase, an acetylase, a glucoronyltransferase, a gluconylepimerase, a sialyltransferase, a mannosyltransferase, a sulfotransferase, a β -acetylgalactosaminyltransferase and a N-acetylglucosaminyl-

transferase.

5. The method of claim 1, wherein said oligosaccharides are selected from the group consisting of galactose α 1-3 galactose β 1-4 glucose, 2'fucosyllactose, 3'fucosyllactose, lacto-N-neo-tetraose, lacto-N-tetraose, lacto-N-fucopentaose, a sialylated derivative of lacto-N-fucopentaose, lacto-N-fucopentaose II, a sialylated derivative of lacto-N-fucopentaose II, lacto-N-fucopentaose III, a sialylated derivative of lacto-N-fucopentaose III, lacto-N-fucopentaose IV, a sialylated derivative of lacto-N-fucopentaose IV, lacto-N-fucopentaose V, a sialylated derivative of lacto-N-fucopentaose V, lacto-N-di-fucopentaose I, a sialylated derivative of lacto-N-di-fucopentaose, lacto-N-di-fucopentaose II, a sialylated derivative of lacto-N-di-fucopentaose II, lacto-N-hexaose, a fucosylated derivative of lacto-N-hexaose, sialyltetrasaccharide a, a fucosylated derivative of sialyltetrasaccharide a, sialyltetrasaccharide b, a fucosylated derivation of sialyltetrasaccharide b, sialyltetrasaccharide c and a fucosylated derivative of sialyltetrasaccharide c.

6. A non-human, transgenic mammal, wherein the genome of said mammal comprises at least one DNA sequence encoding an enzyme operatively linked to a mammary gland-specific promoter, wherein expression of said at least one DNA sequence results in the production of oligosaccharides and glycoproteins in the milk of said mammal.

7. The non-human, transgenic mammal of claim 6, wherein said mammal is selected from the group consisting of a mouse, a rat, a rabbit, a pig, a goat, a sheep and a cow.

8. The non-human, transgenic mammal of claim 6, wherein said enzyme is a glycosyltransferase.

9. The non-human, transgenic mammal of claim 8, wherein said glycosyltransferase is selected from the group consisting of a fucosyltransferase, a galactosyltransferase, an acetylase, a glucoronyltransferase, a gluconylepimerase, a
 5 sialyltransferase, a mannosyltransferase, a sulfotransferase, a β -acetylgalactosaminyltransferase and a N-acetylglucosaminyltransferase.

10. The non-human, transgenic mammal of claim 6, wherein
 10 said oligosaccharides are selected from the group consisting of galactose α 1-3 galactose β 1-4 glucose, 2'fucosyllactose, 3'fucosyllactose, lacto-N-neo-tetraose, lacto-N-tetraose, lacto-N-fucopentaose, a sialylated derivative of lacto-N-fucopentaose, lacto-N-fucopentaose II, a sialylated derivative of lacto-N-
 15 fucopentaose II, lacto-N-fucopentaose III, a sialylated derivative of lacto-N-fucopentaose III, lacto-N-fucopentaose IV, a sialylated derivative of lacto-N-fucopentaose IV, lacto-N-fucopentaose V, a sialylated derivative of lacto-N-fucopentaose V, lacto-N-di-fucopentaose I, a sialylated derivative of lacto-
 20 N-di-fucopentaose, lacto-N-di-fucopentaose II, a sialylated derivative of lacto-N-di-fucopentaose II, lacto-N-hexaose, a fucosylated derivative of lacto-N-hexaose, sialyltetrasaccharide a, a fucosylated derivative of sialyltetrasaccharide a, sialyltetrasaccharide b, a fucosylated derivative of
 25 sialyltetrasaccharide b, sialyltetrasaccharide c and a fucosylated derivative of sialyltetrasaccharide c.

11. A method of producing milk in a non-human, transgenic mammal, said method comprising the steps of:
 30 (a) preparing at least one transgene, said at least one transgene comprising is operable association 1) a promoter functional in mammary secretory cells, 2) a DNA sequence encoding an enzyme, wherein expression of said at least one transgene results in production of oligosaccharides and
 35 glycoproteins in said milk of said mammal;

(b) inserting said at least one transgene into said non-human, transgenic mammal;

(c) milking said non-human, transgenic mammal in order to obtain said milk, wherein said milk comprises said
5 oligosaccharides and glycoproteins.

12. The method of claim 11, wherein said non-human, transgenic mammal is selected from the group consisting of a mouse, a rat, a rabbit, a pig, a goat, a sheep and a cow.
10

13. The method of claim 11, wherein said enzyme is a glycosyltransferase.

14. The method of claim 13, wherein said
15 glycosyltransferase is selected from the group consisting of a fucosyltransferase, a galactosyltransferase, an acetylase, a glucoronyltransferase, a gluconylepimerase, a sialyltransferase, a mannosyltransferase, a sulfotransferase, a β -acetylgalactosaminyltransferase and a N-acetylglucosaminyl-
20 transferase.

15. The method of claim 11, wherein said oligosaccharides are selected from the group consisting of galactose α 1-3 galactose β 1-4 glucose, 2'fucosyllactose, 3'fucosyllactose,
25 lacto-N-neo-tetraose, lacto-N-tetraose, lacto-N-fucopentaose, a sialylated derivative of lacto-N-fucopentaose, lacto-N-fucopentaose II, a sialylated derivative of lacto-N-fucopentaose II, lacto-N-fucopentaose III, a sialylated derivative of lacto-N-fucopentaose III, lacto-N-fucopentaose IV, a sialylated
30 derivative of lacto-N-fucopentaose IV, lacto-N-fucopentaose V, a sialylated derivative of lacto-N-fucopentaose V, lacto-N-di-fucopentaose I, a sialylated derivative of lacto-N-di-fucopentaose II, a sialylated derivative of lacto-N-di-fucopentaose II, lacto-N-hexaose, a
35 fucosylated derivative of lacto-N-hexaose, sialyltetrasaccharide

a, a fucosylated derivative of sialyltetrasaccharide a, sialyltetrasaccharide b, a fucosylated derivation of sialyltetrasaccharide b, sialyltetrasaccharide c and a fucosylated derivative of sialyltetrasaccharide c.

5

16. A milk produced by a non-human, transgenic mammal, wherein the genome of said non-human, transgenic mammal comprises at least one DNA sequence encoding an enzyme, said DNA sequence being operatively linked to a promoter, wherein
10 expression of said at least one DNA sequence results in the production of oligosaccharides and glycoproteins.

17. The milk of claim 16, wherein said mammal is selected from the group consisting of a mouse, a rat, a rabbit, a pig, a
15 goat, a sheep and a cow.

18. The milk of claim 17, wherein said enzyme is a glycosyltransferase.

19. The milk of claim 18, wherein said glycosyltransferase
20 is selected from the group consisting of a fucosyltransferase, a galactosyltransferase, an acetylase, a glucuronyltransferase, a gluconylepimerase, a sialyltransferase, a mannosyltransferase, a sulfotransferase, a β -acetylgalactosaminyltransferase and a N-
25 acetylglucosaminyl-transferase.

20. The milk of claim 17, wherein said oligosaccharides are selected from the group consisting of galactose α 1-3 galactose β 1-4 glucose, 2'fucosyllactose, 3'fucosyllactose,
30 lacto-N-neo-tetraose, lacto-N-tetraose, lacto-N-fucopentaose, a sialylated derivative of lacto-N-fucopentaose, lacto-N-fucopentaose II, a sialylated derivative of lacto-N-fucopentaose II, lacto-N-fucopentaose III, a sialylated derivative of lacto-N-fucopentaose III, lacto-N-fucopentaose IV, a sialylated
35 derivative of lacto-N-fucopentaose IV, lacto-N-fucopentaose V, a

sialylated derivative of lacto-N-fucopentaose V, lacto-N-di-fucopentaose I, a sialylated derivative of lacto-N-di-fucopentaose, lacto-N-di-fucopentaose II, a sialylated derivative of lacto-N-di-fucopentaose II, lacto-N-hexaose, a
5 fucosylated derivative of lacto-N-hexaose, sialyltetrasaccharide a, a fucosylated derivative of sialyltetrasaccharide a, sialyltetrasaccharide b, a fucosylated derivative of sialyltetrasaccharide b, sialyltetrasaccharide c and a fucosylated derivative of sialyltetrasaccharide c.

10

21. A fraction of said milk of claim 18, wherein said fraction comprises at least one oligosaccharide, at least one glycoprotein or a combination thereof.

15

22. An oligosaccharide or glycoprotein purified from the milk of claim 18.

20

23. A nutritional product comprising said milk of claim 18, said fraction of claim 21 or said oligosaccharide or glycoprotein of claim 22.

24. The nutritional product of claim 23, wherein said product is an infant formula.

25

25. The nutritional product of claim 24, wherein said infant formula is in the form of a liquid or a solid.

30

26. A method of providing nutrition to patients comprising administering to said patients said milk of claim 18, said fraction of claim 21 or said oligosaccharide or glycoprotein of claim 22 in an amount sufficient to effect said nutrition.

1/26

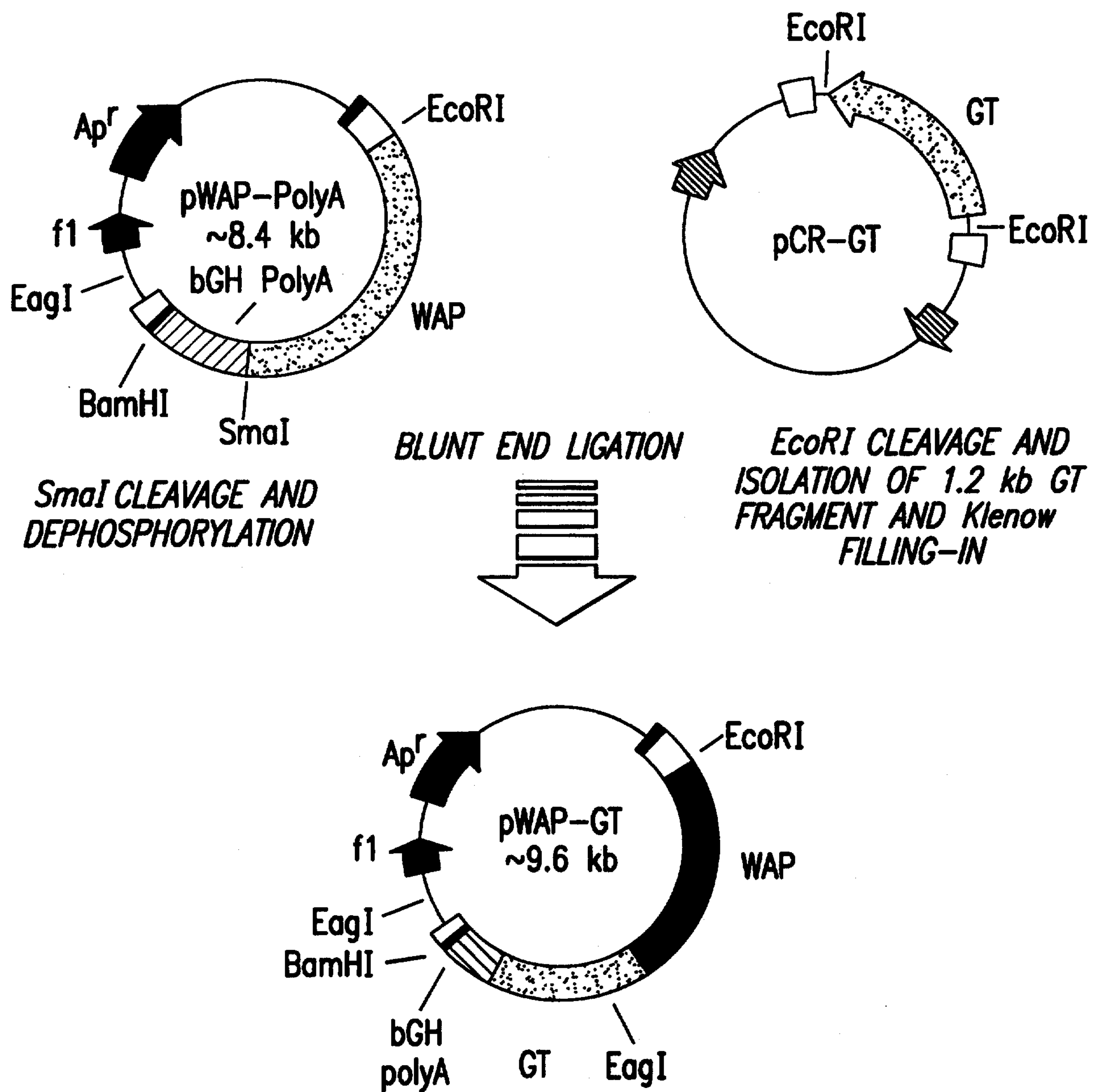


FIG.1

2/26

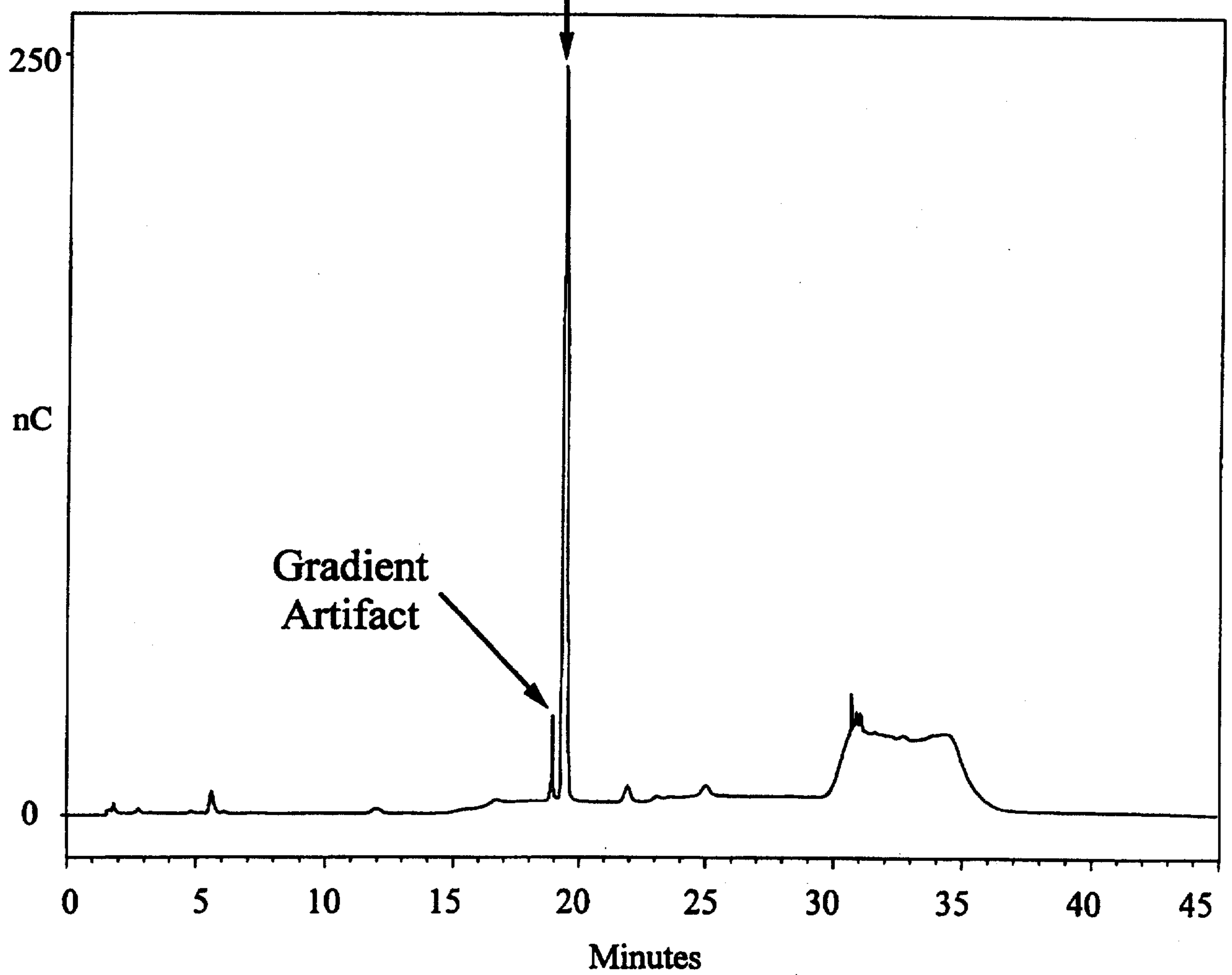
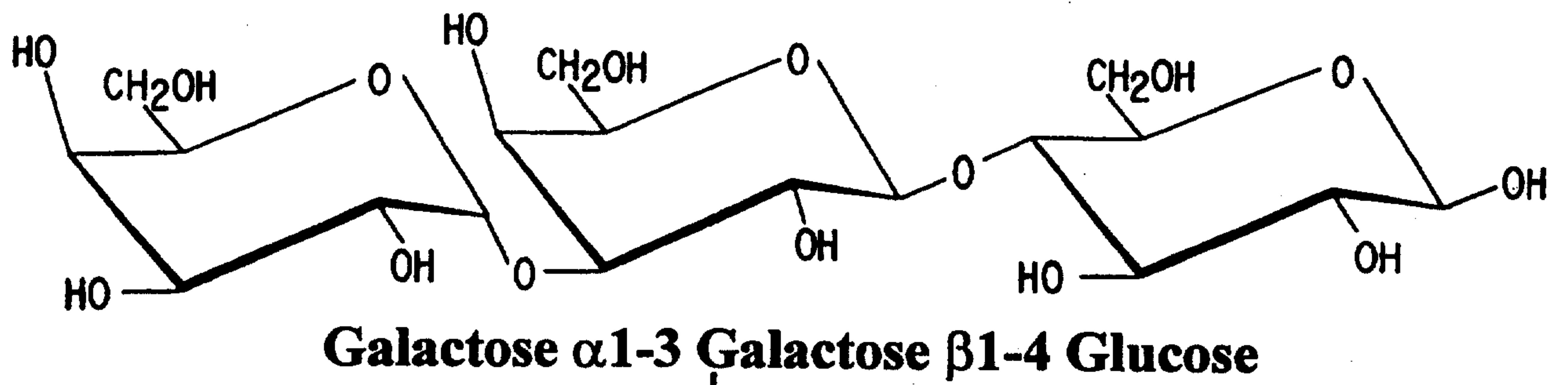


FIG. 2

3/26

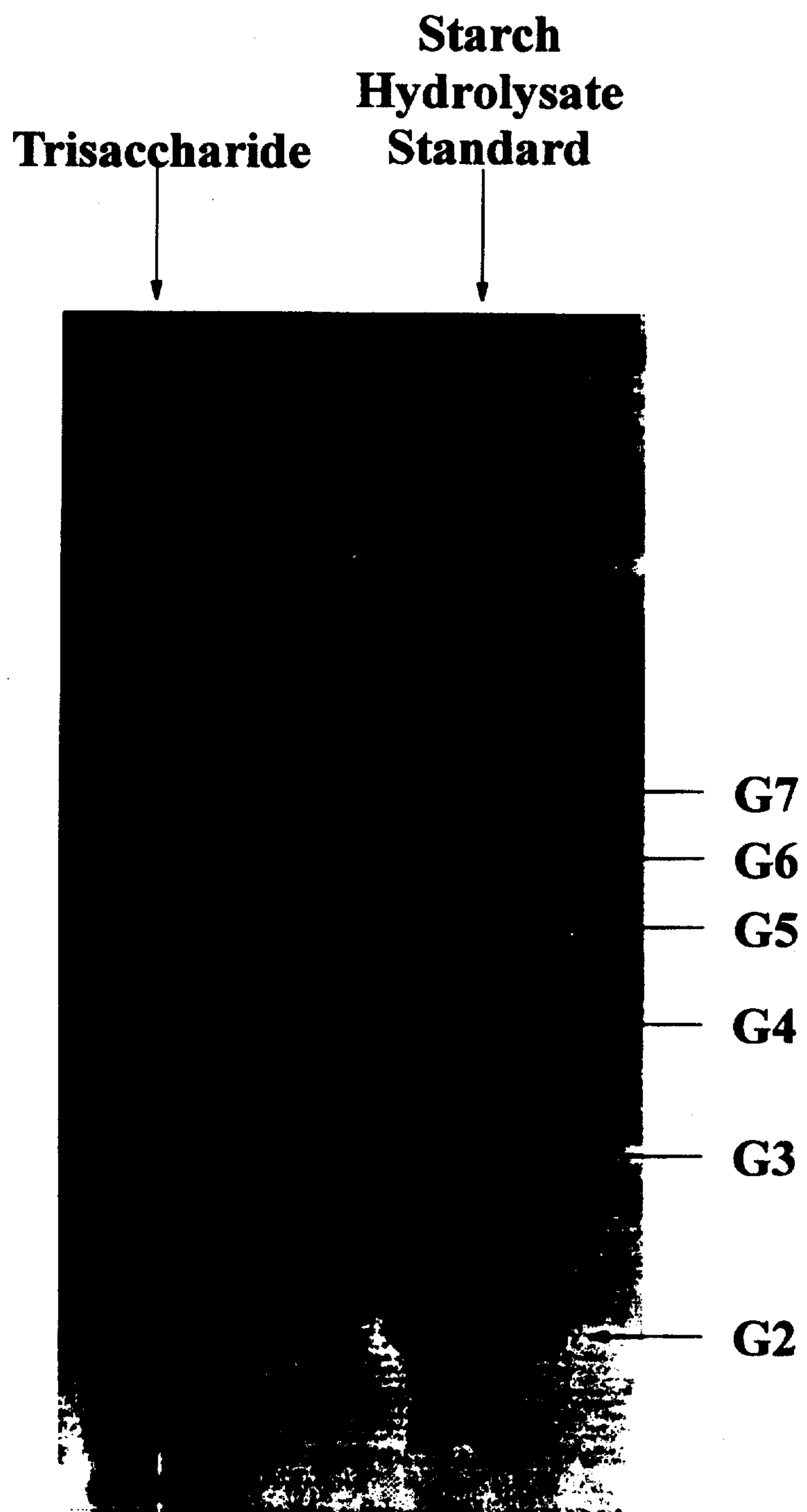


FIG.3

4/26

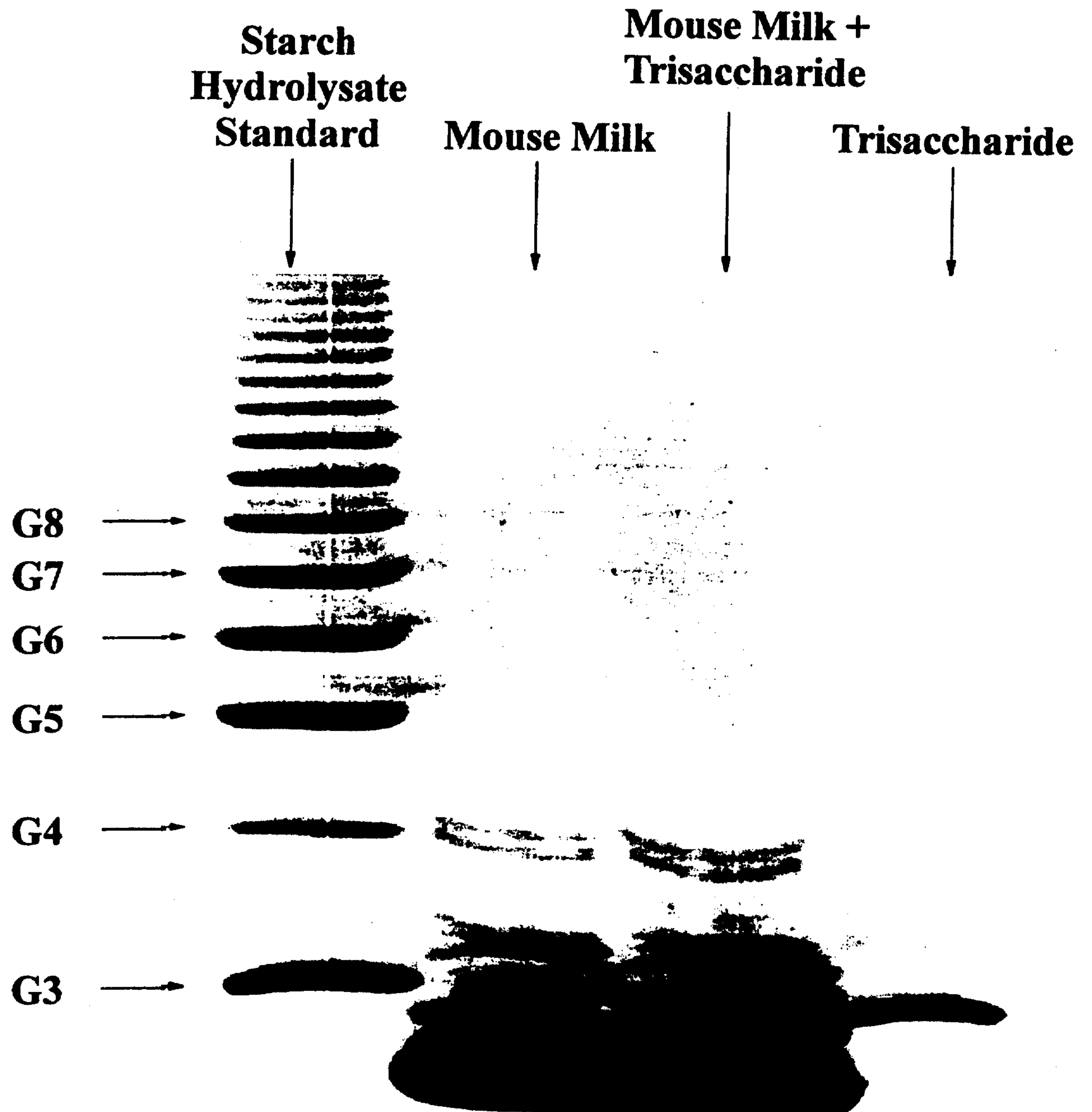


FIG.4

5/26

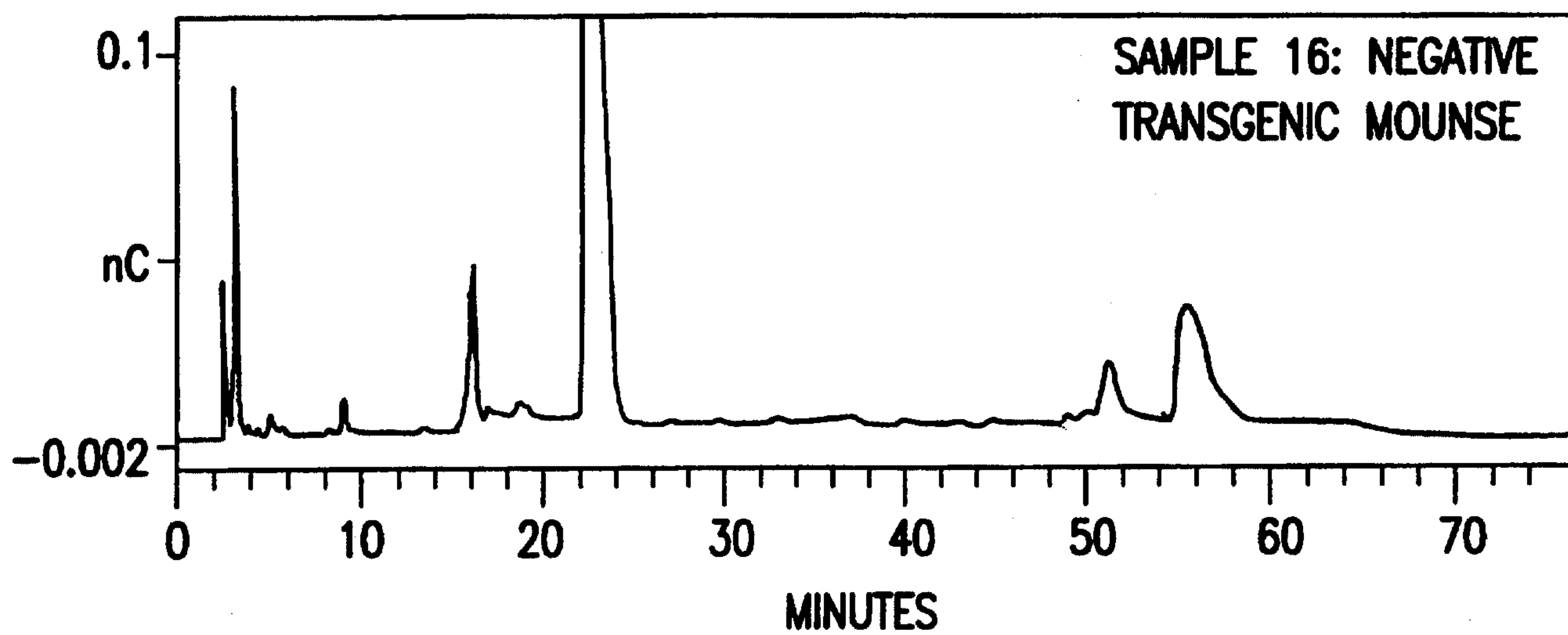


FIG.5A

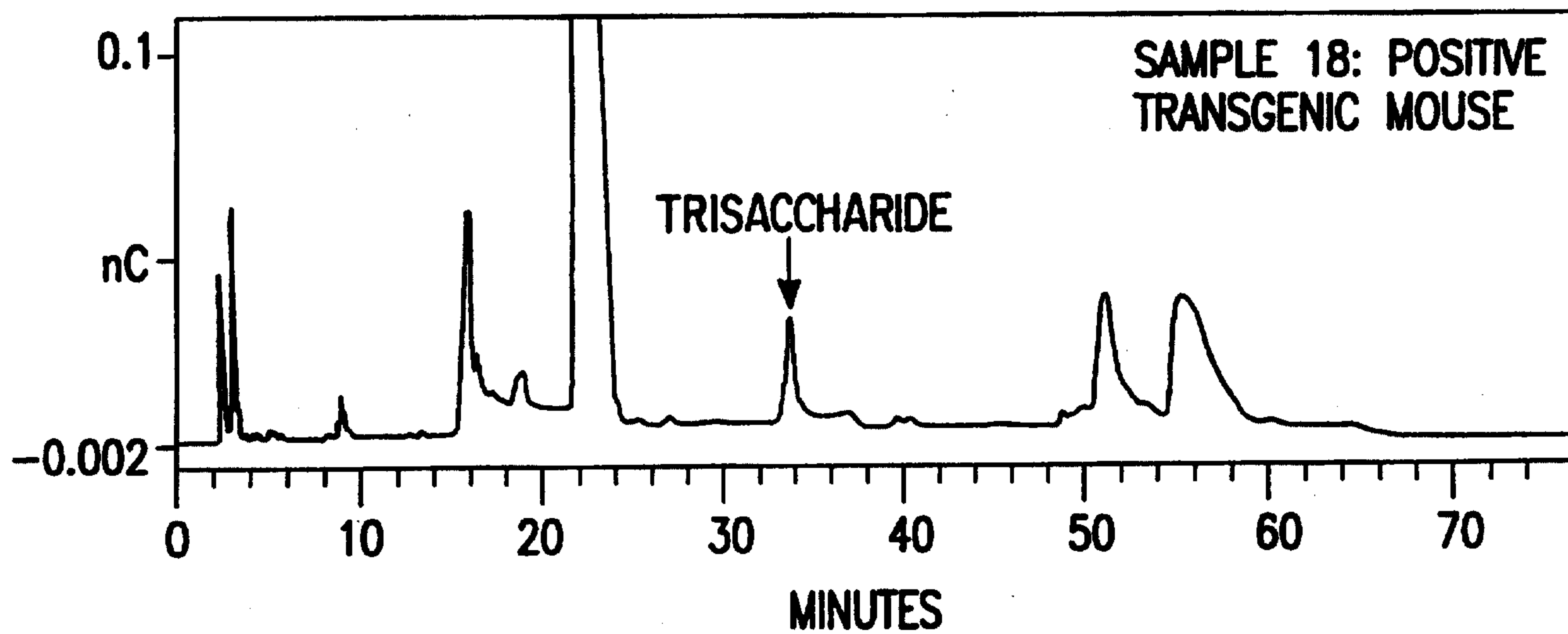


FIG.5B

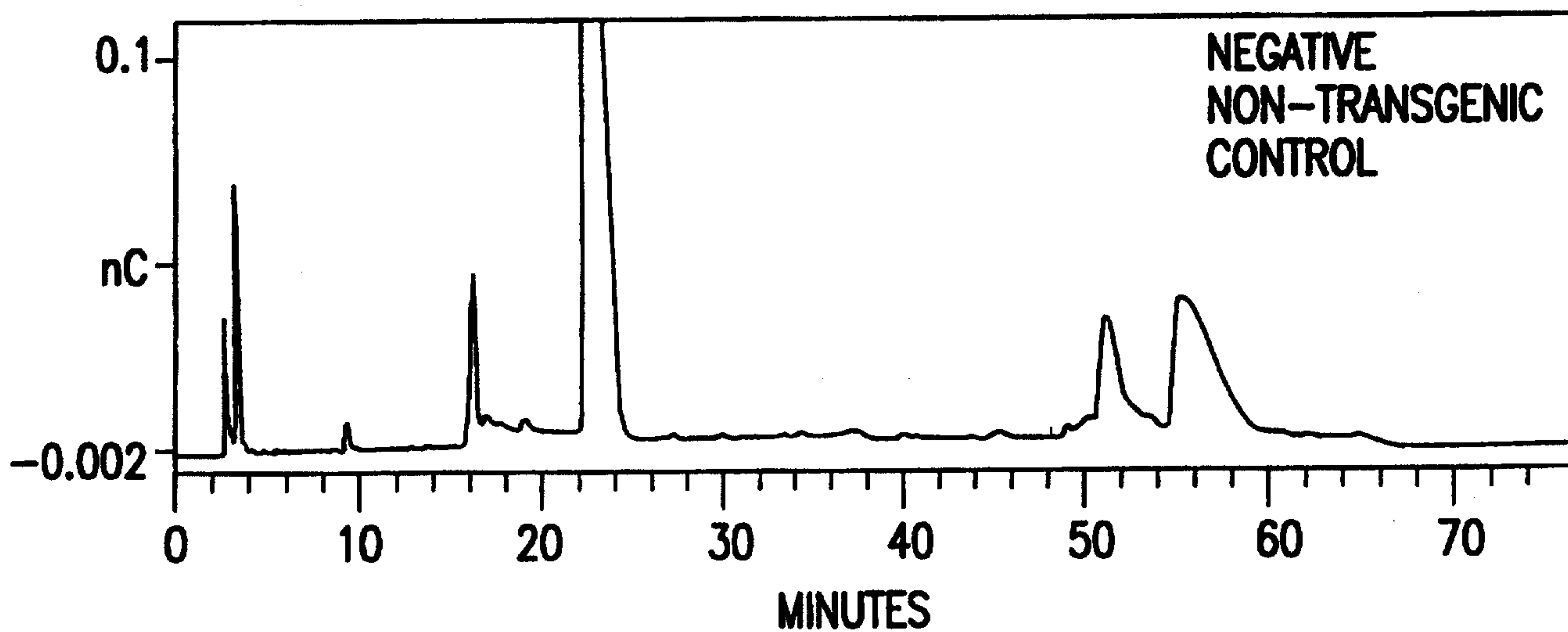


FIG.5C

6/26

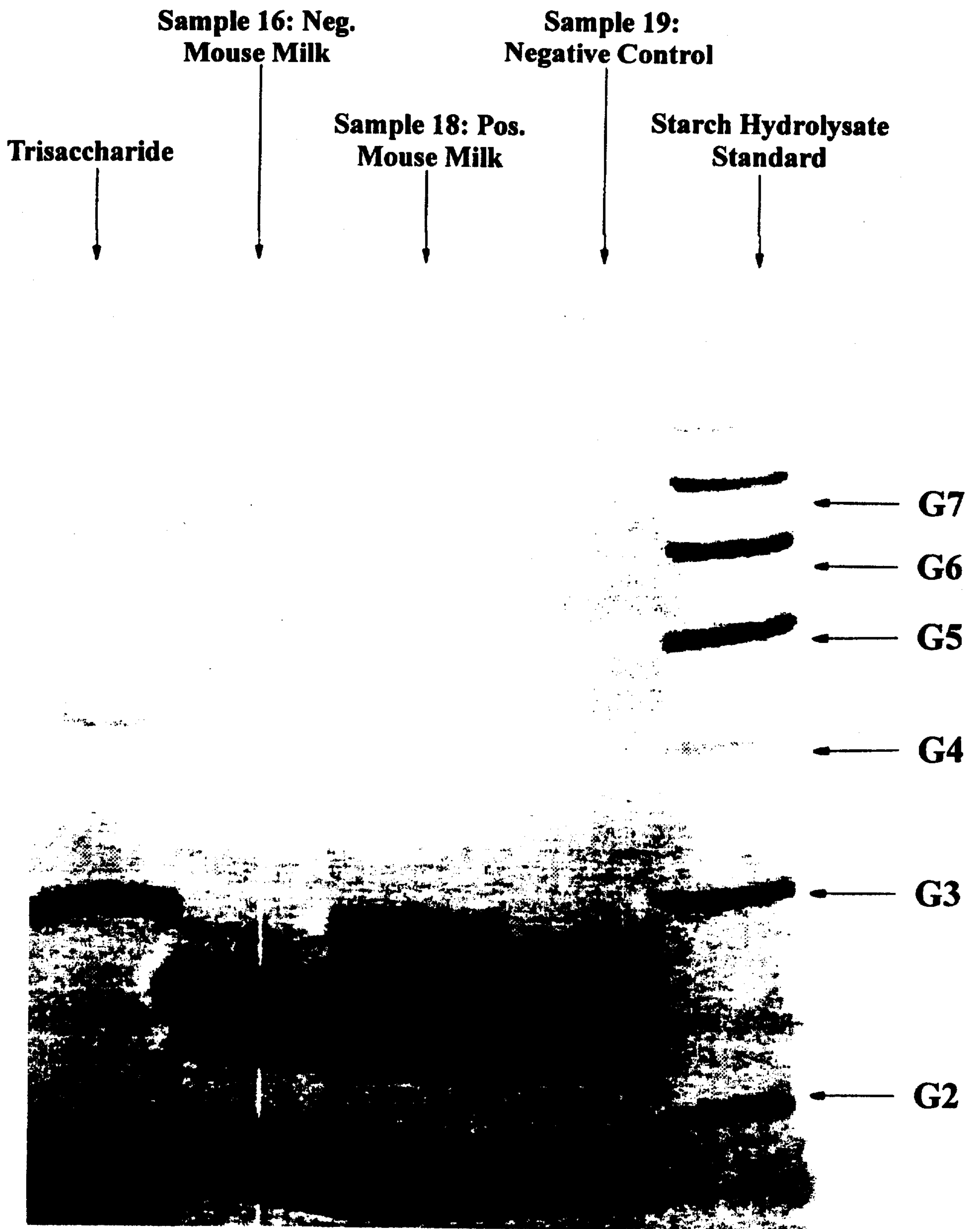


FIG.6

7/26

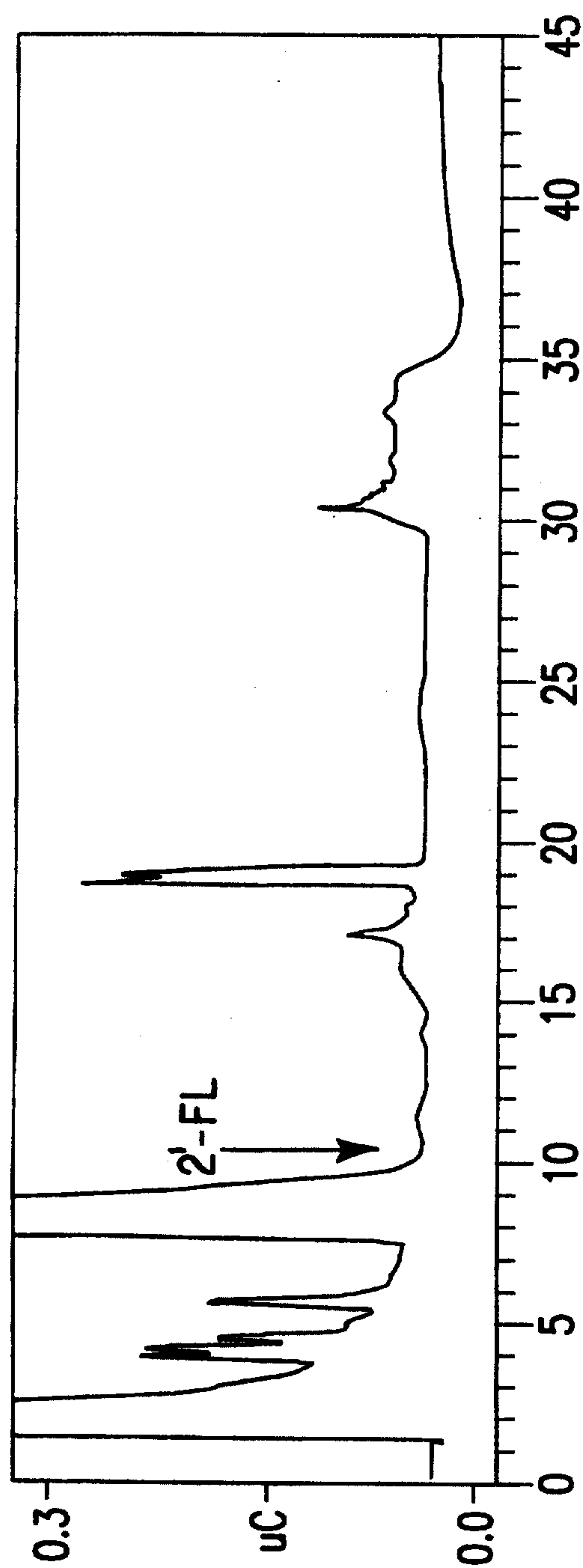


FIG. 7A

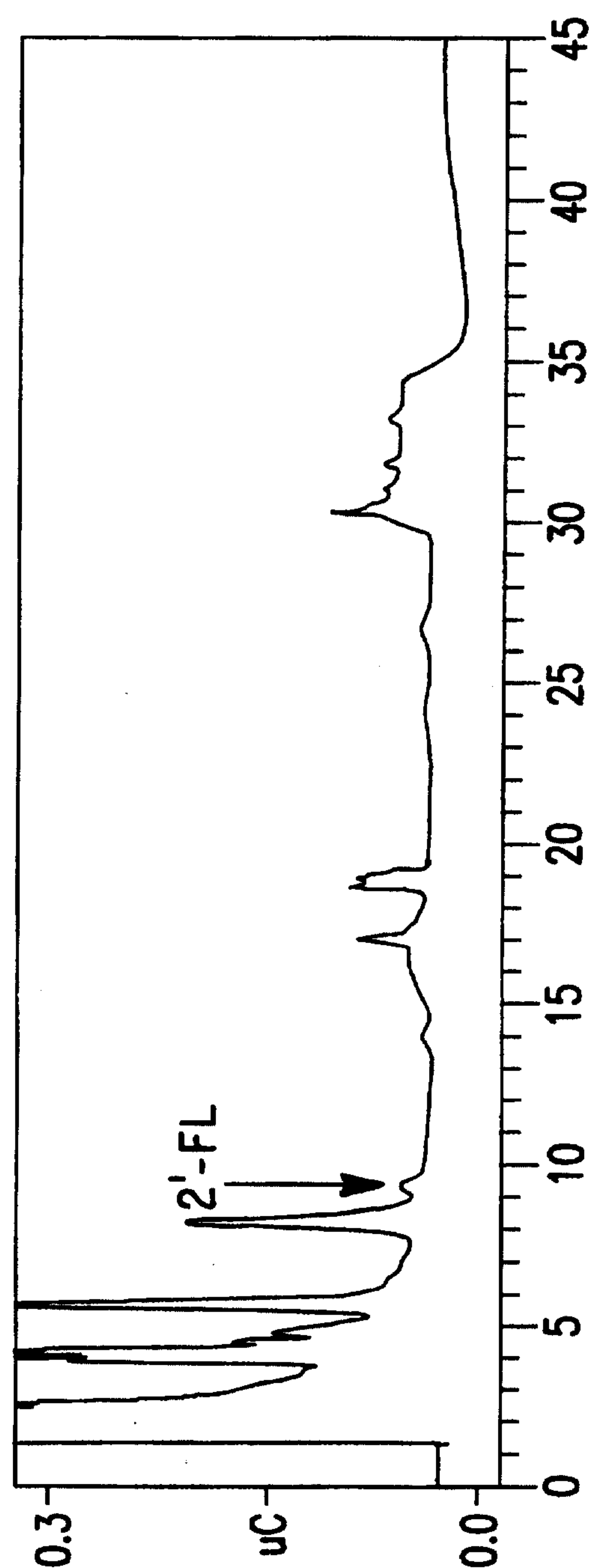


FIG. 7B

8/26

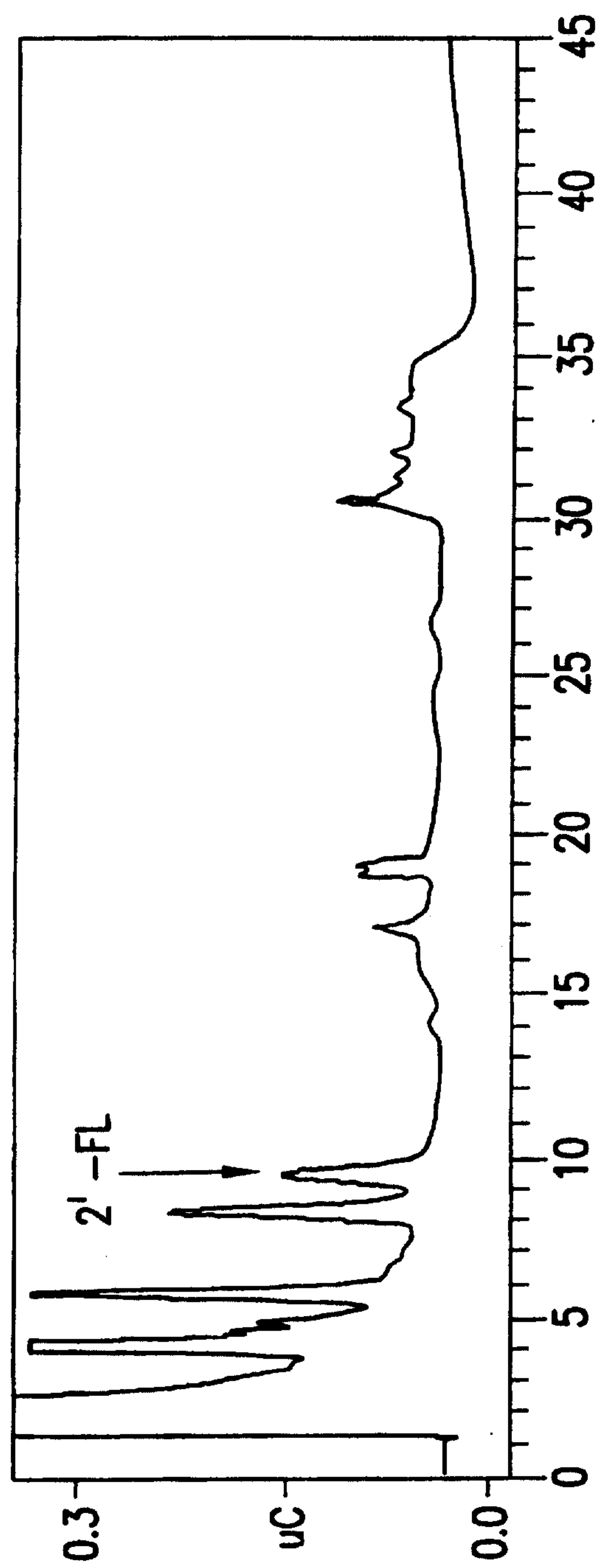


FIG.7C

9/26

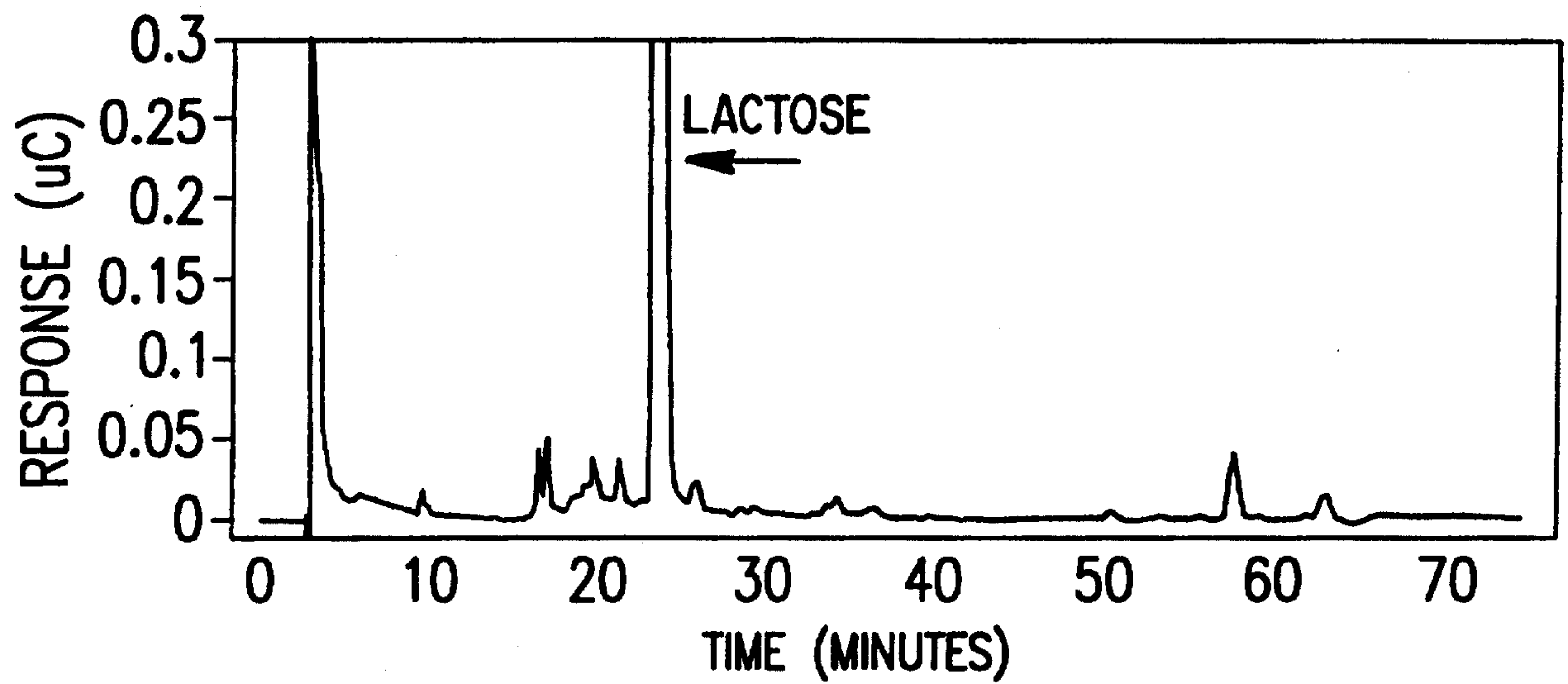


FIG. 8A

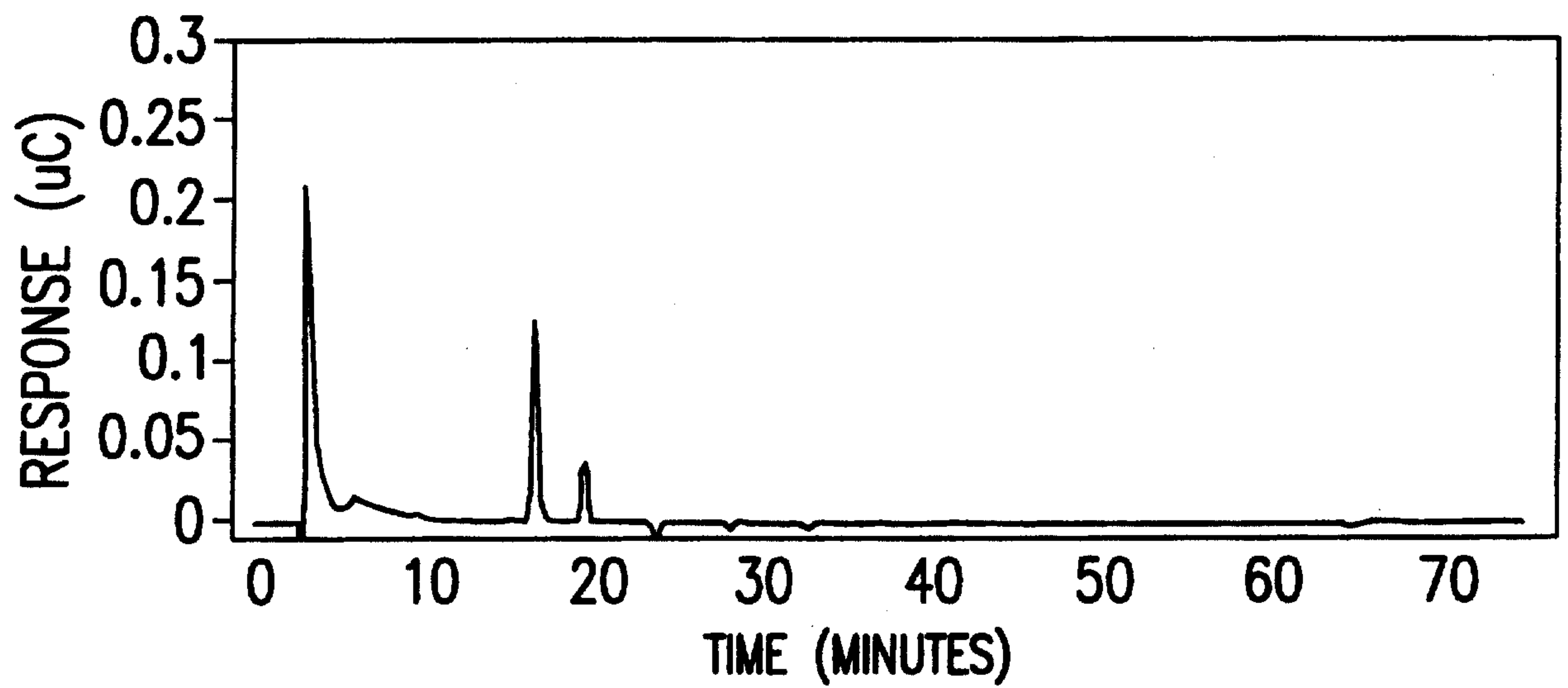


FIG. 8B

10/26

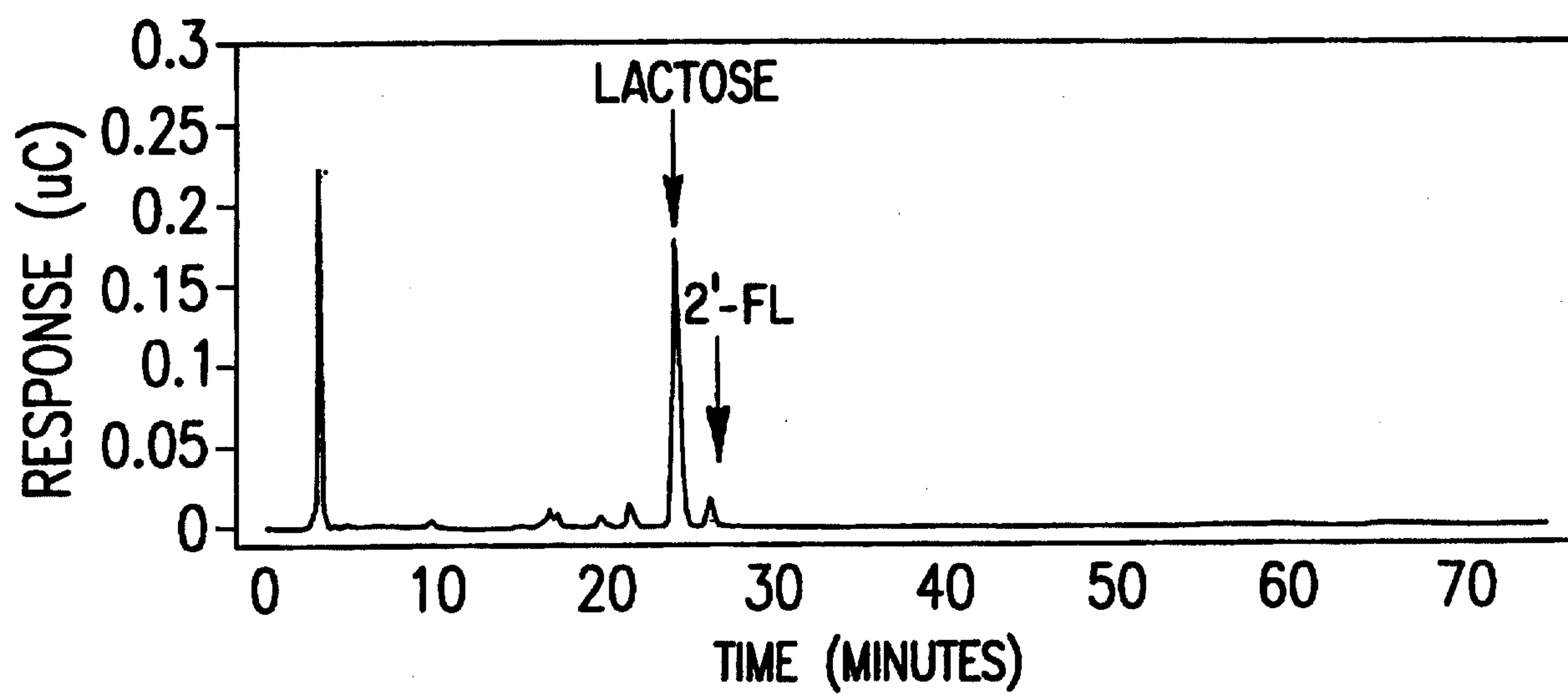


FIG. 8C

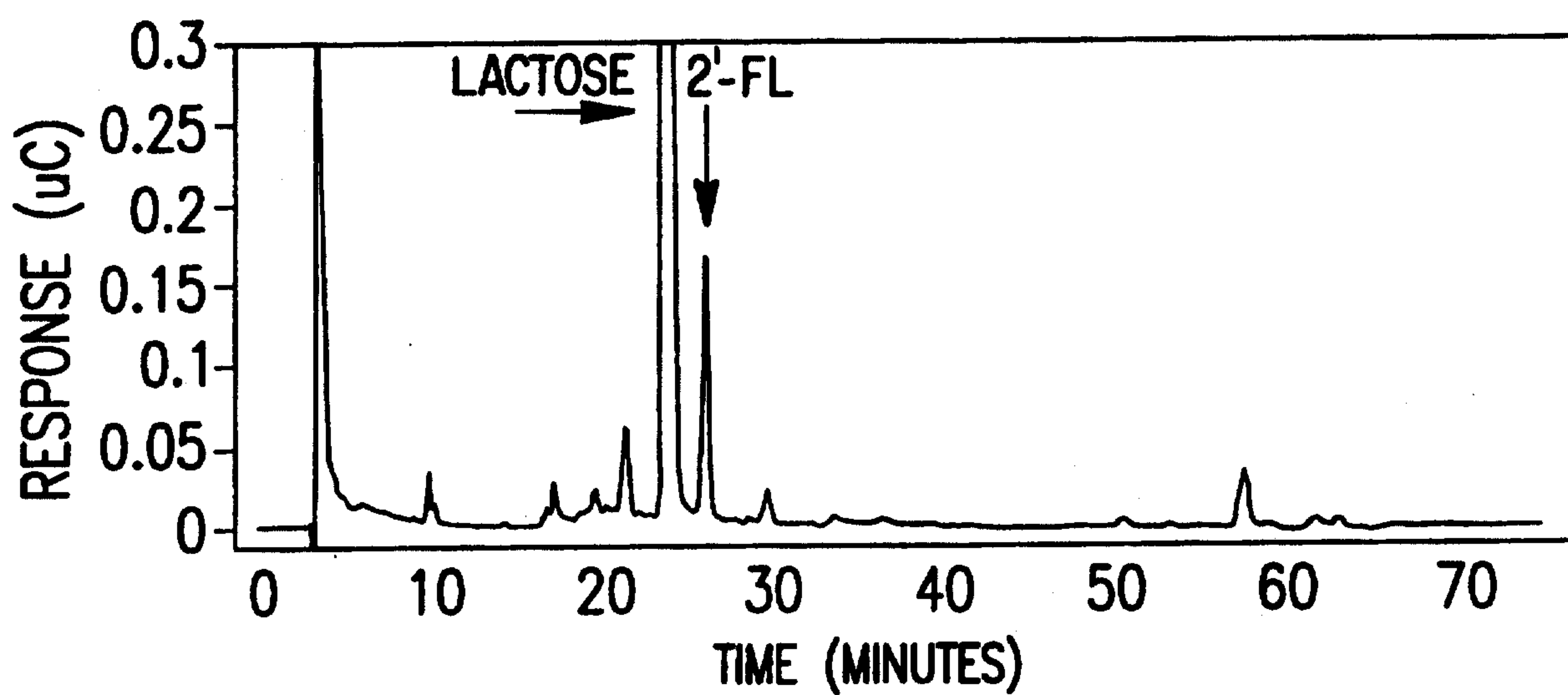


FIG. 8D

11/26

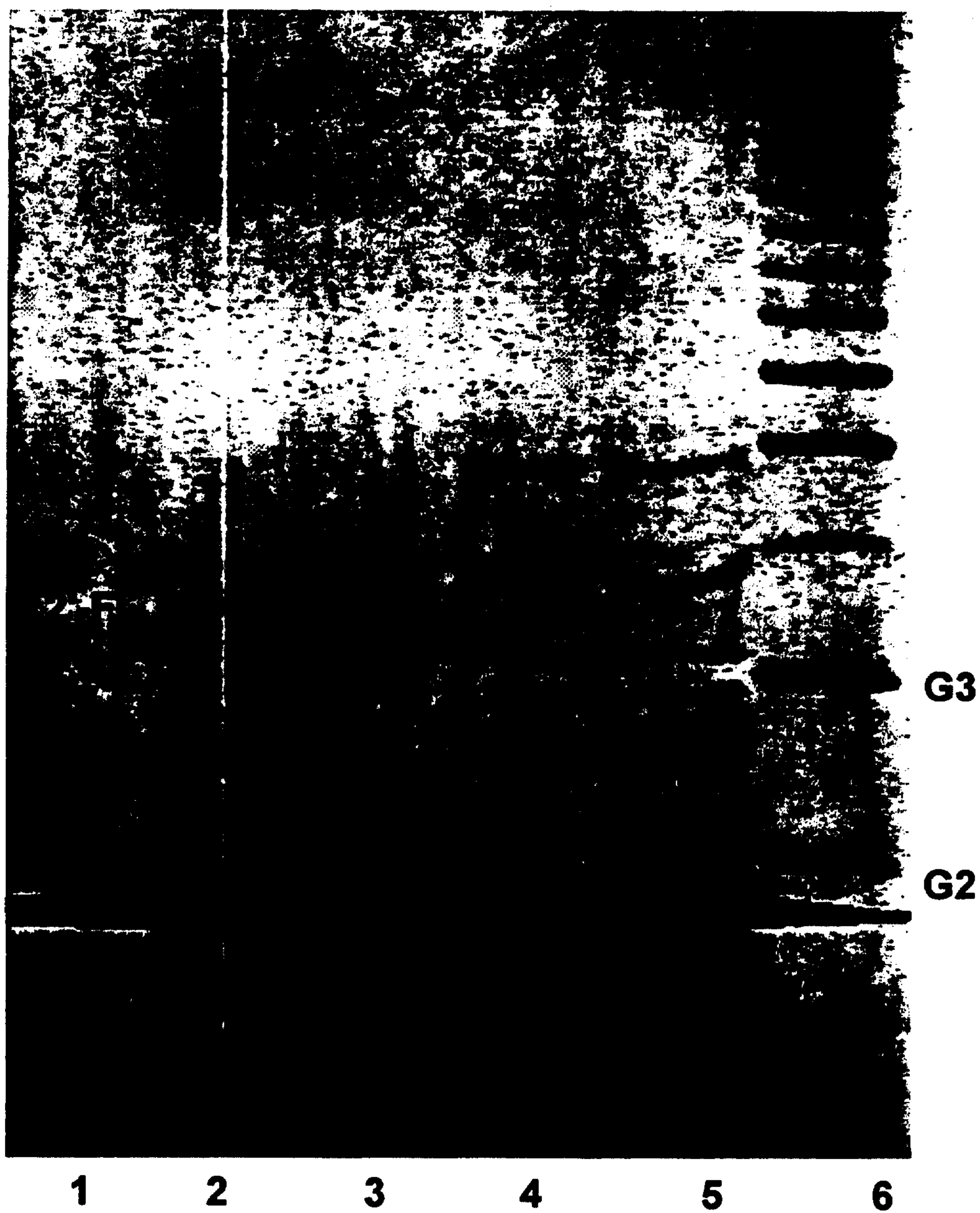


FIG.9

12/26

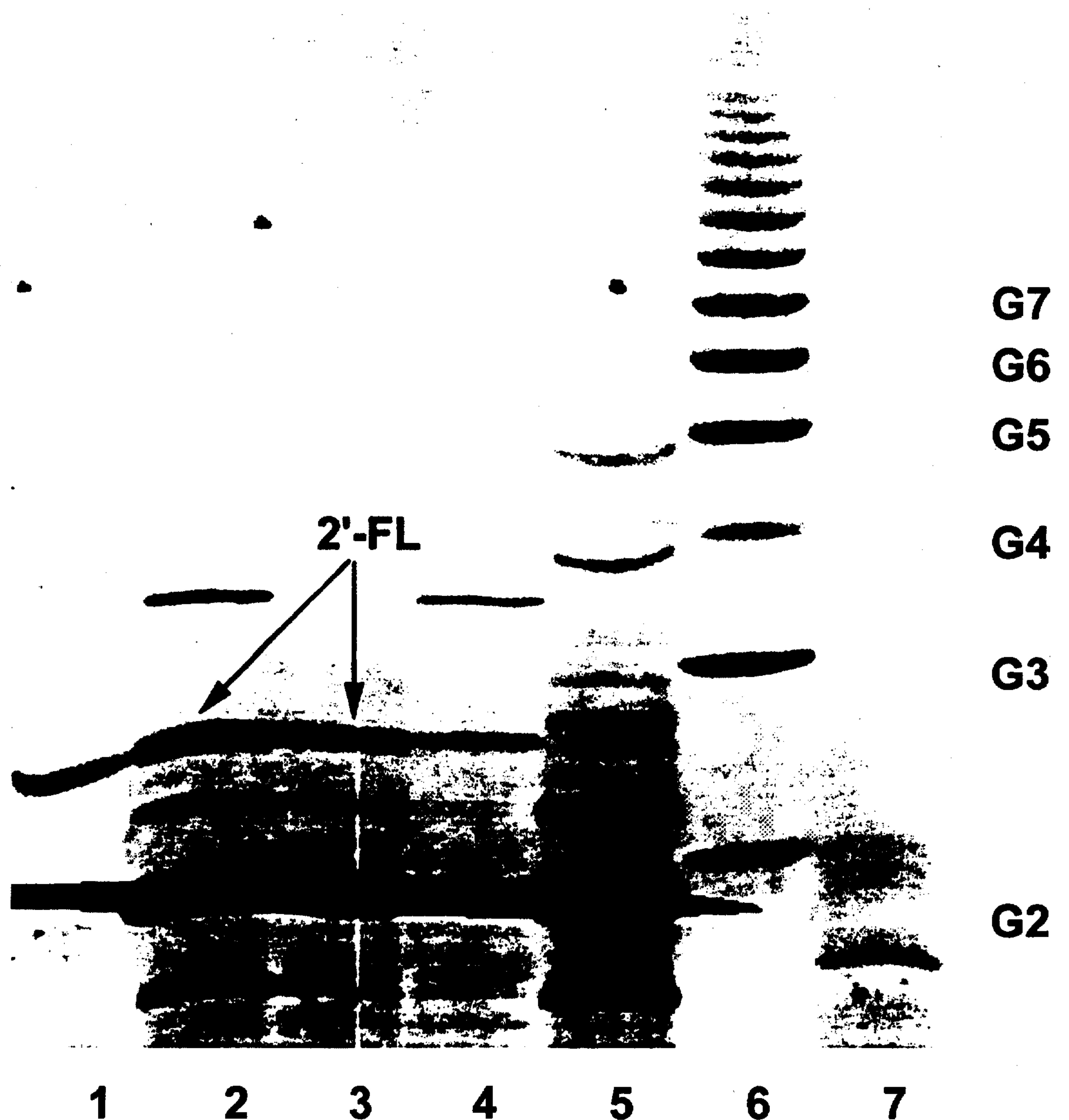


FIG.10

13/26

1 2 3 4



FIG. 11

14/26

1 2 3 4 S



FIG.12

15/26

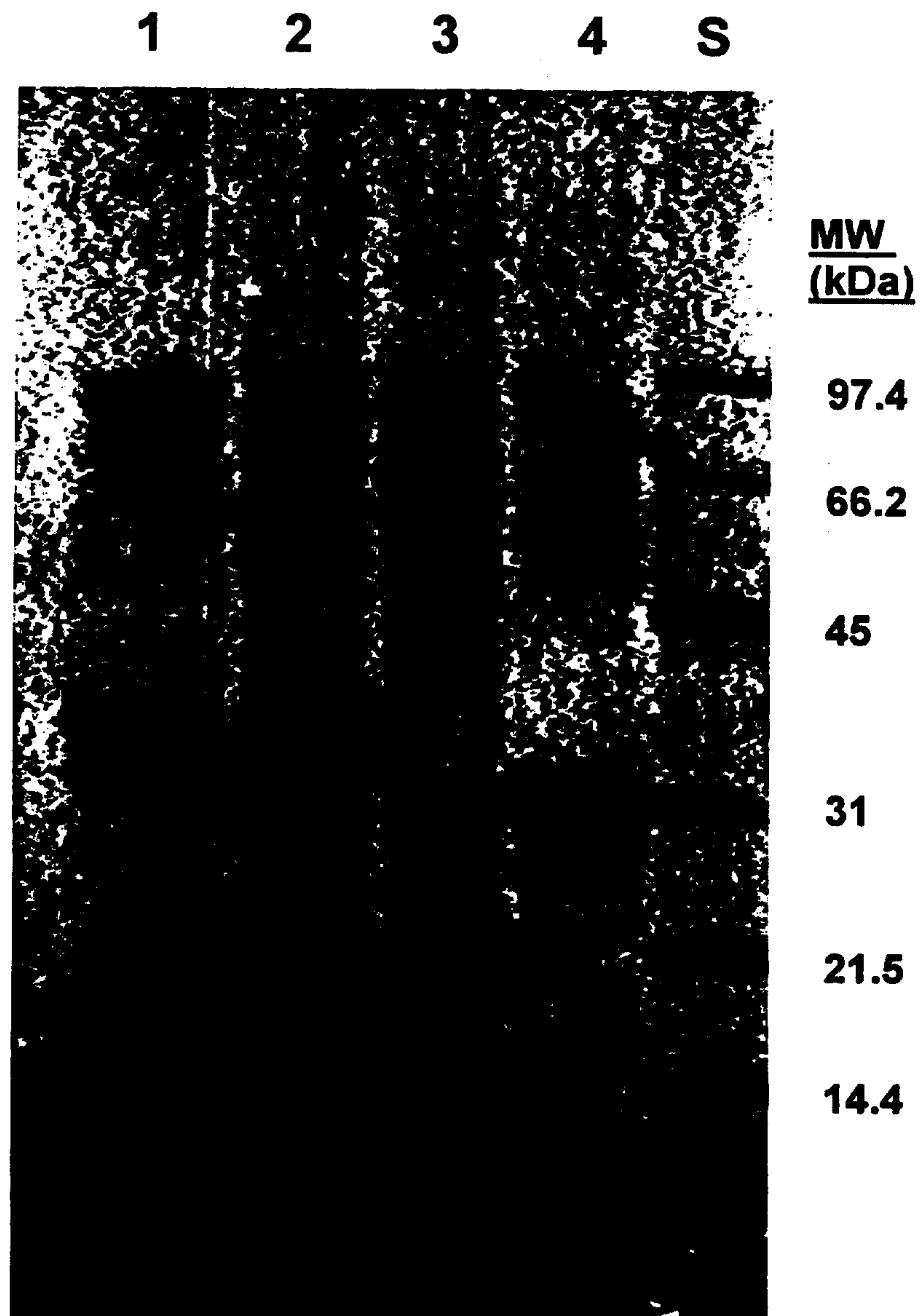


FIG.13

16/26

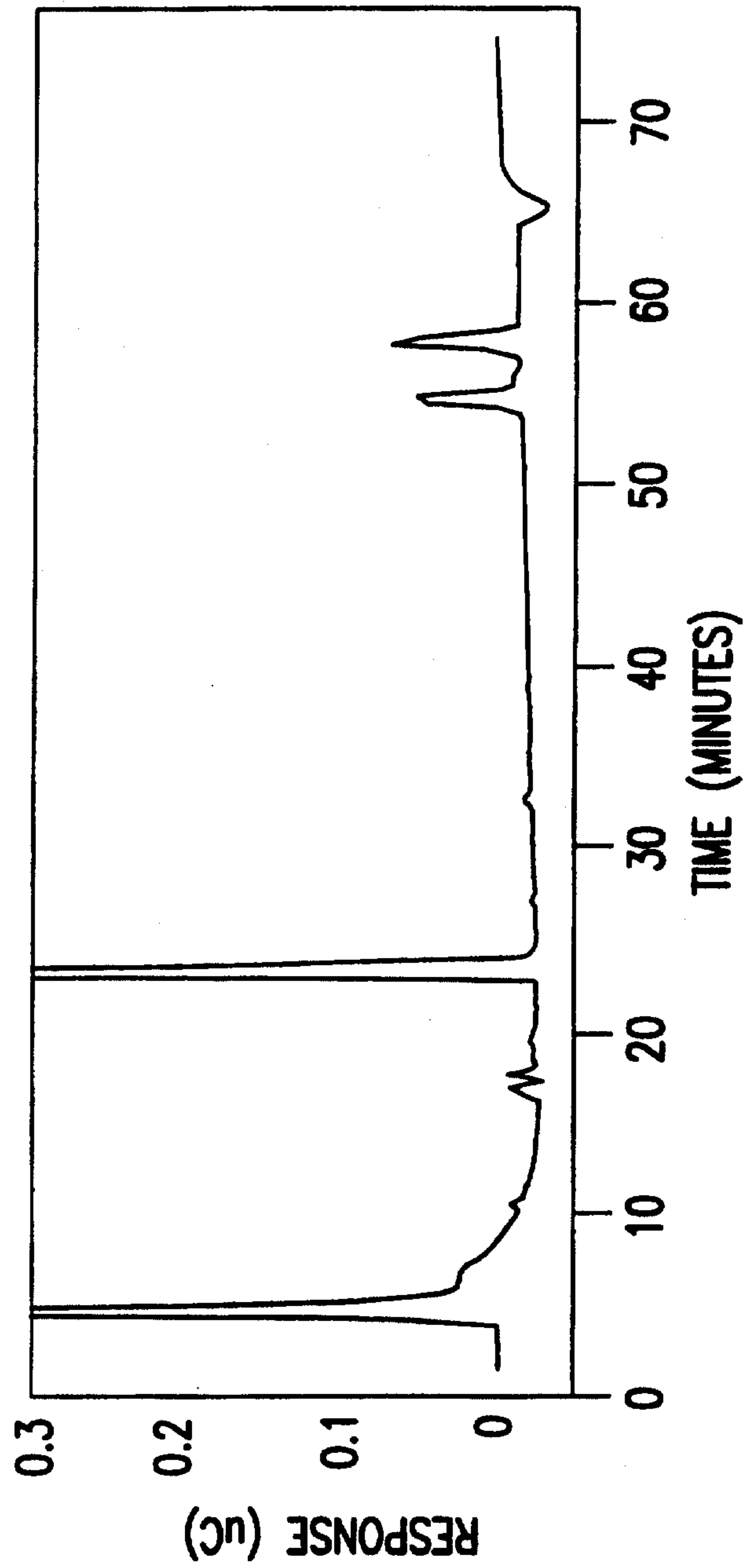


FIG. 14A

17/26

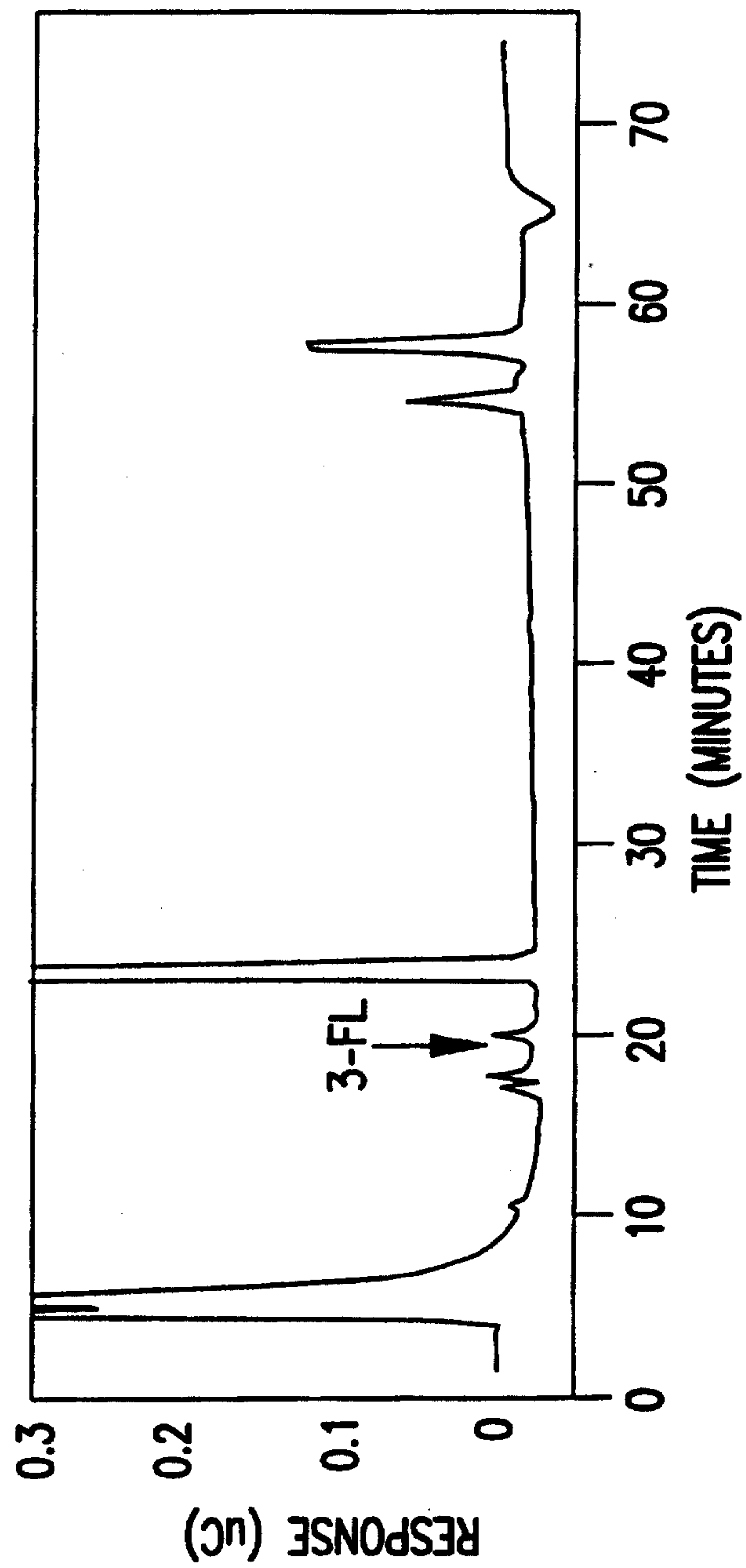


FIG. 14B

18/26

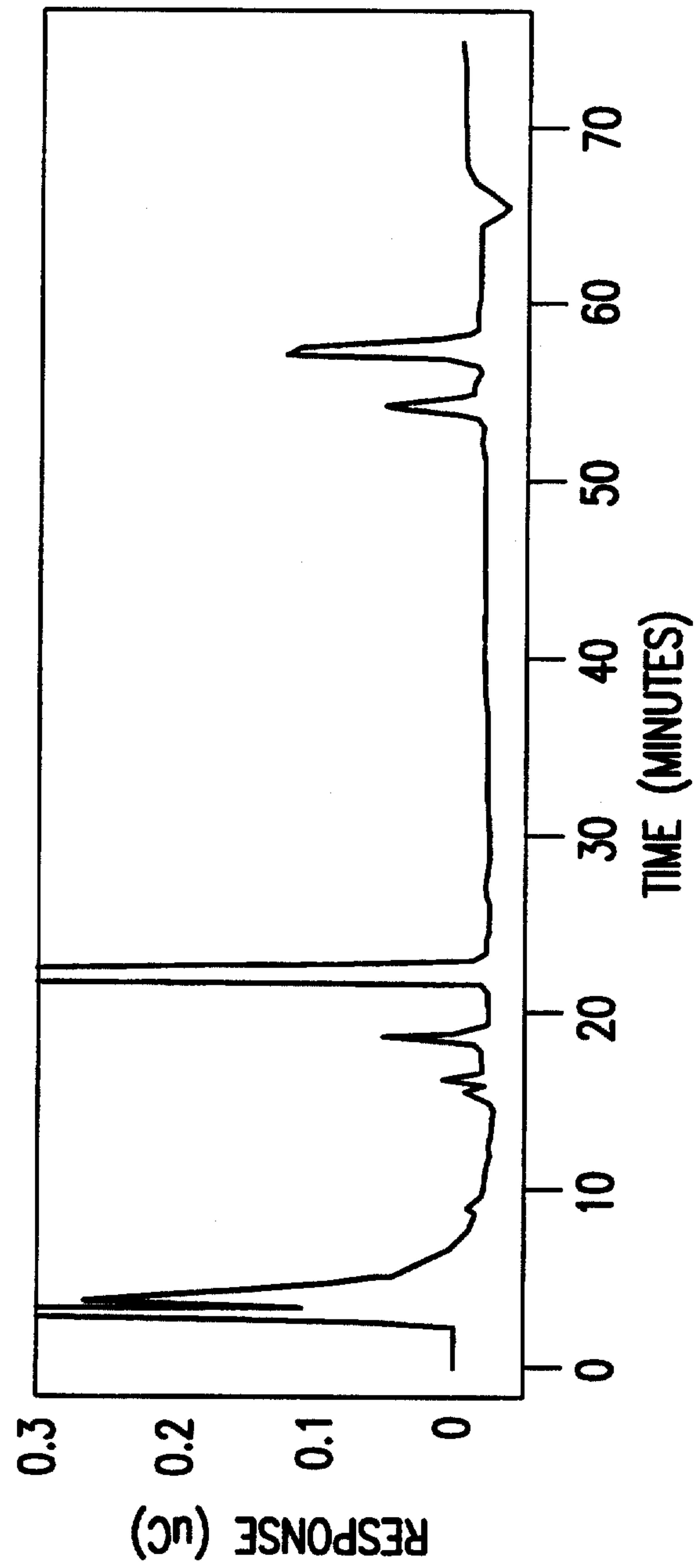


FIG.14C

19/26

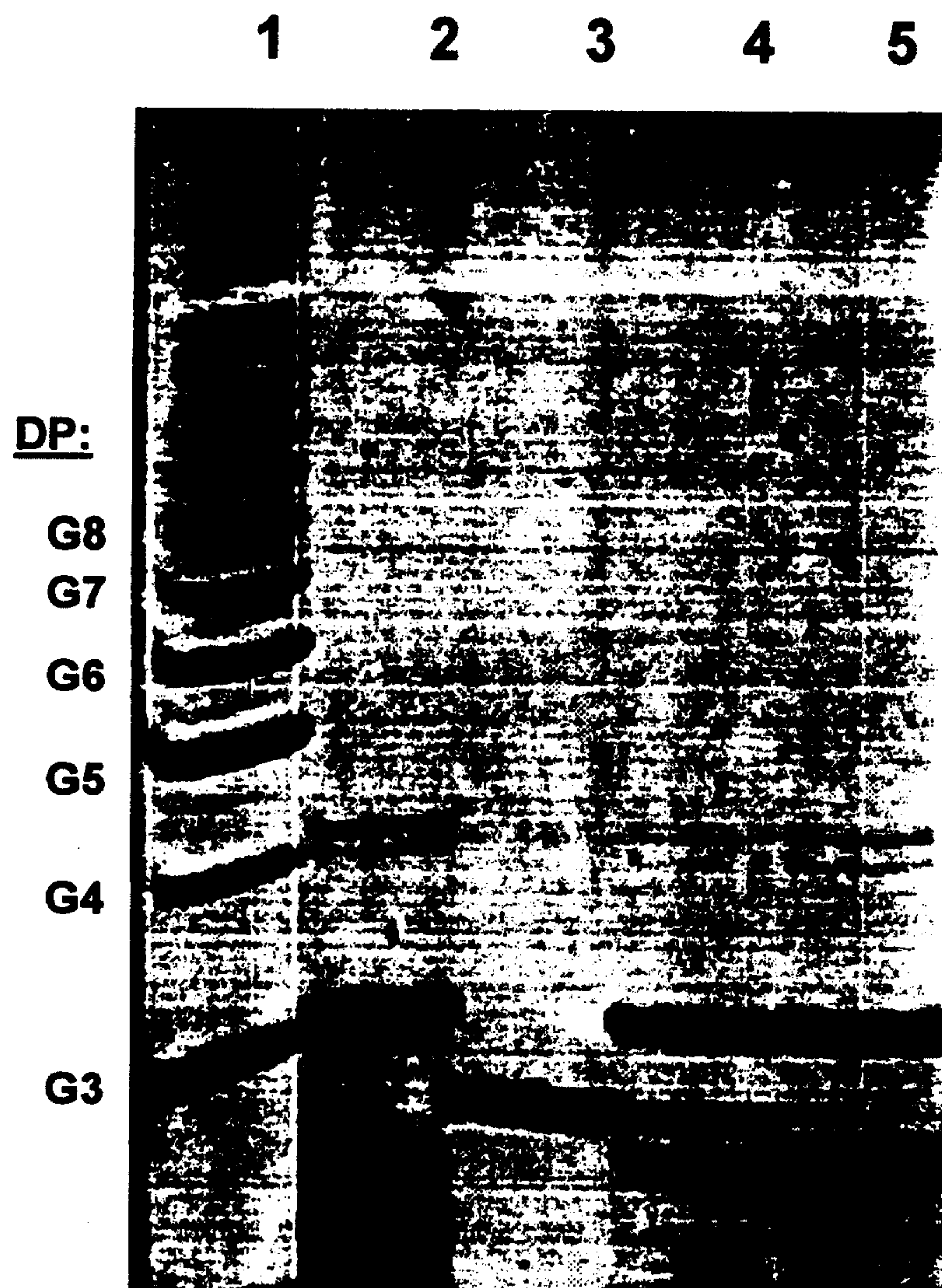


FIG.15

20/26

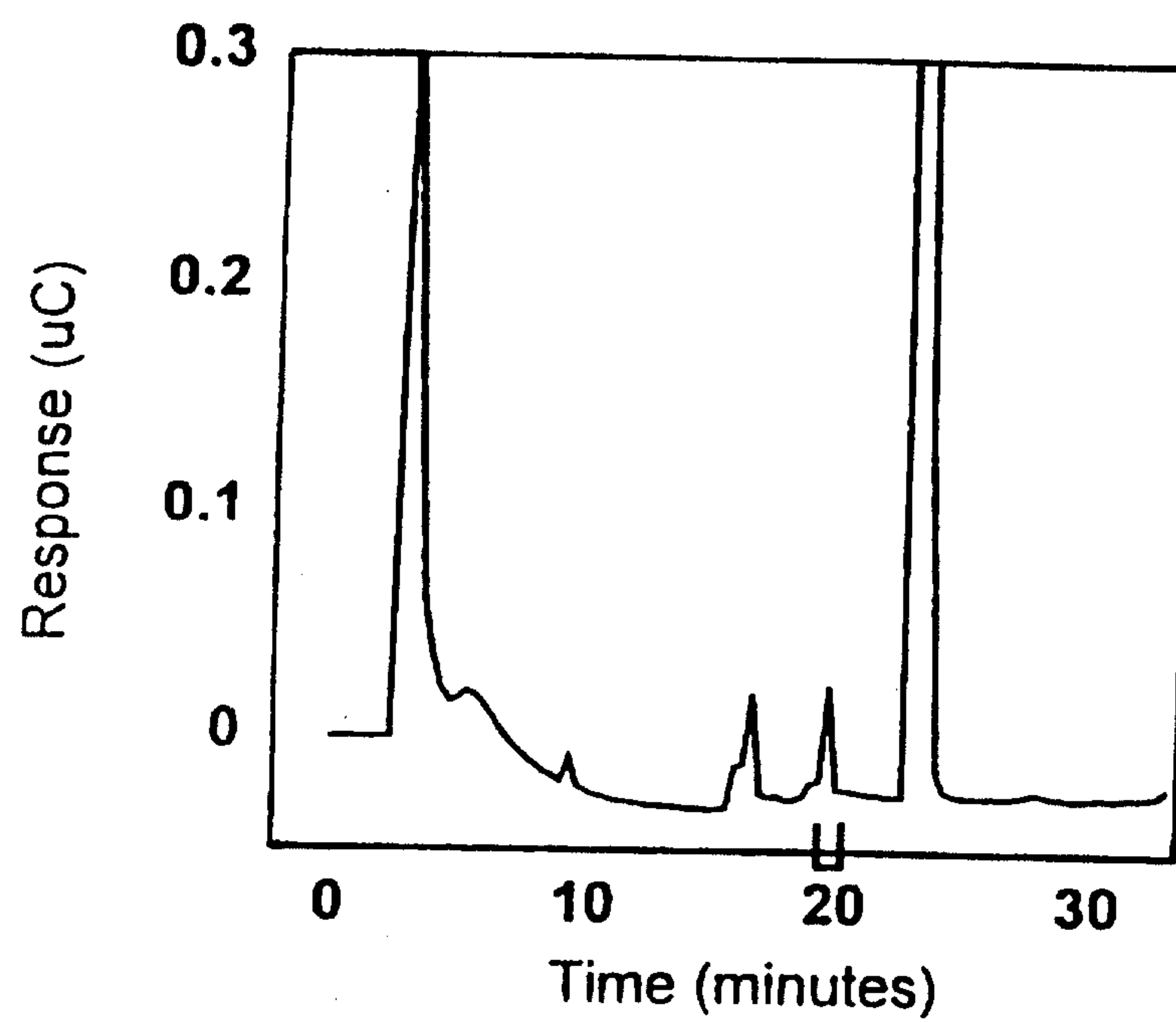


FIG. 16A

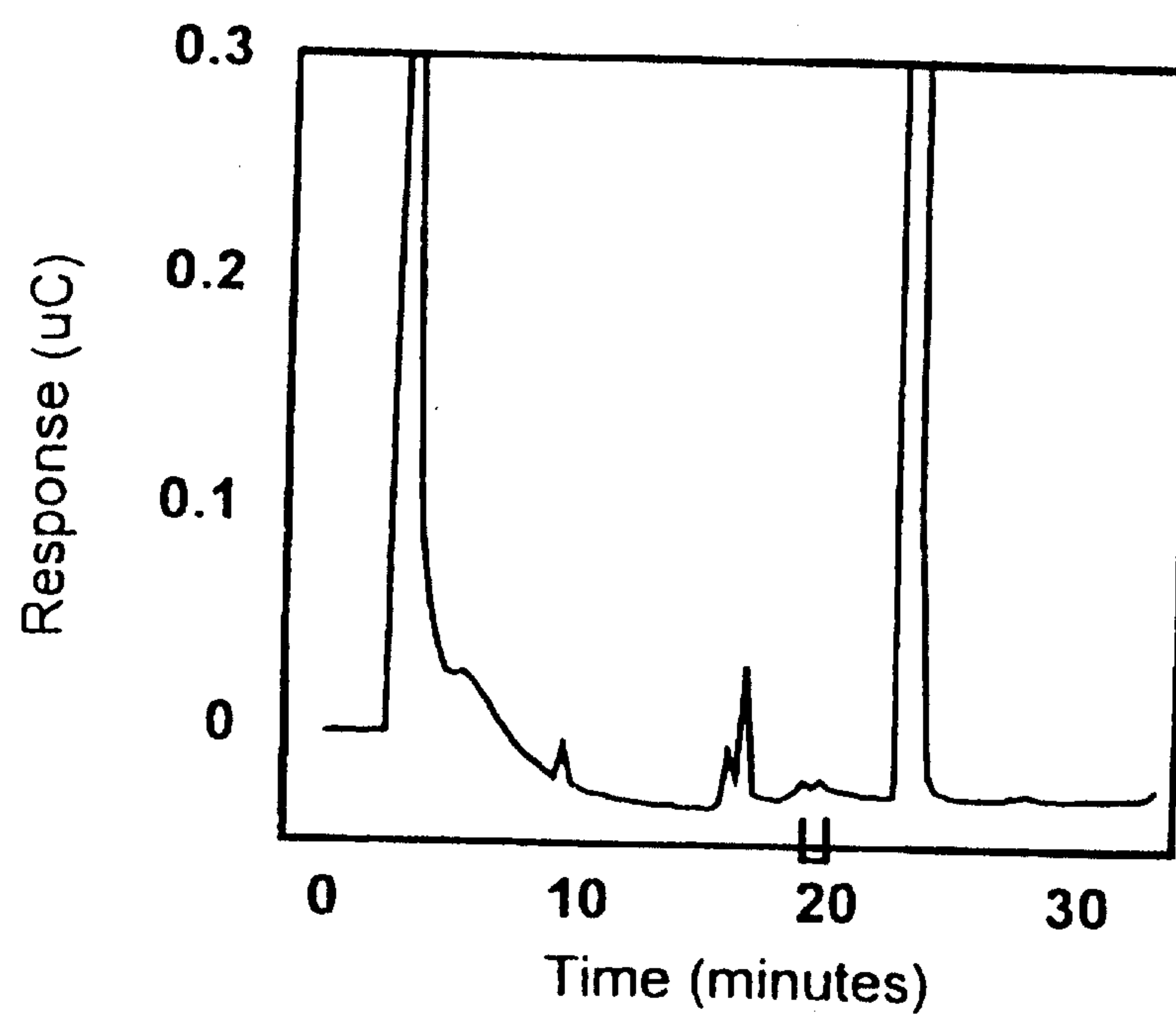


FIG. 16B

21/26

1 2 3 4 5 6 7 8



DP:

G6

G5

G4

G3

FIG.16C

22/26

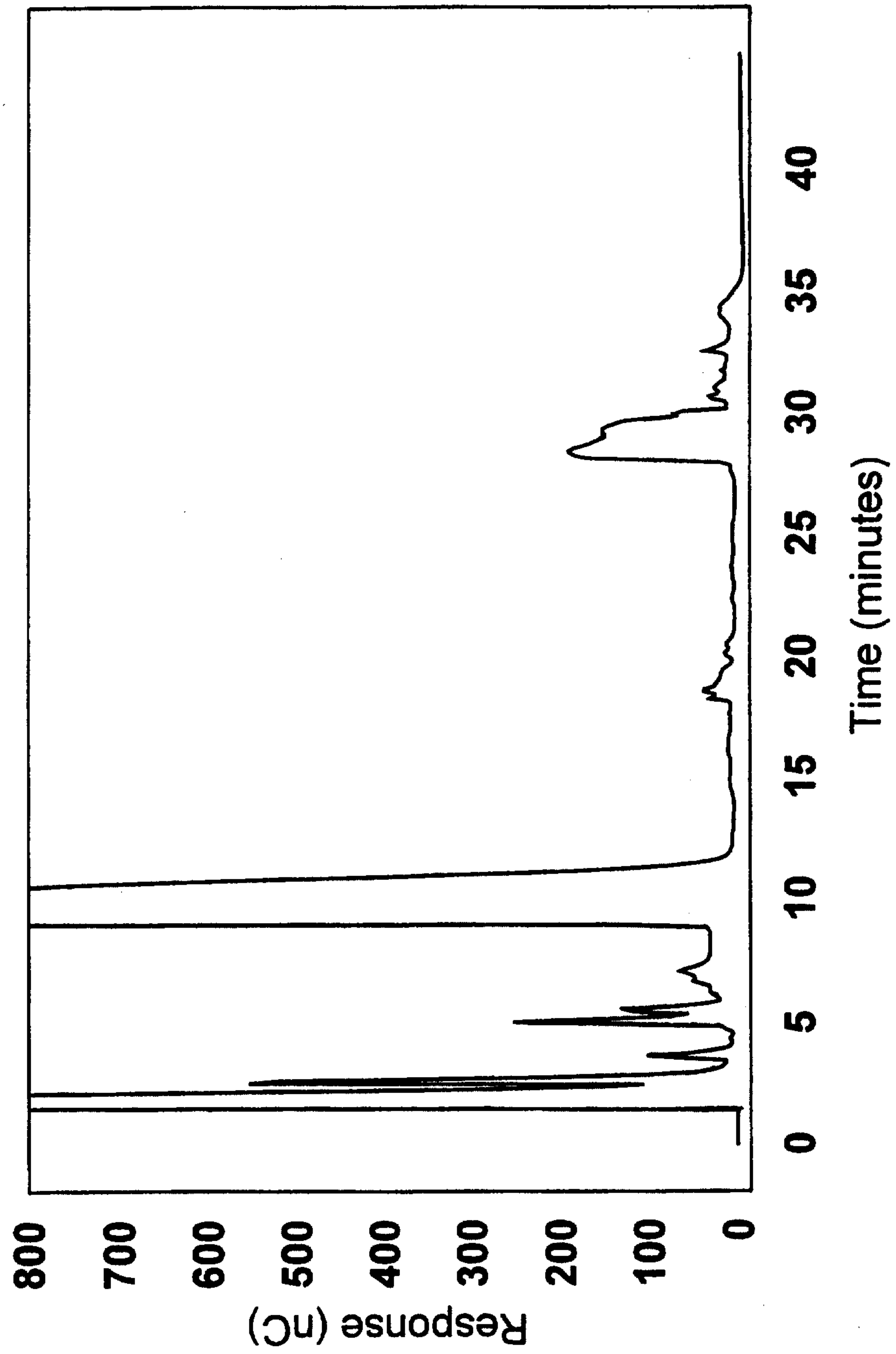


FIG.17

23/26

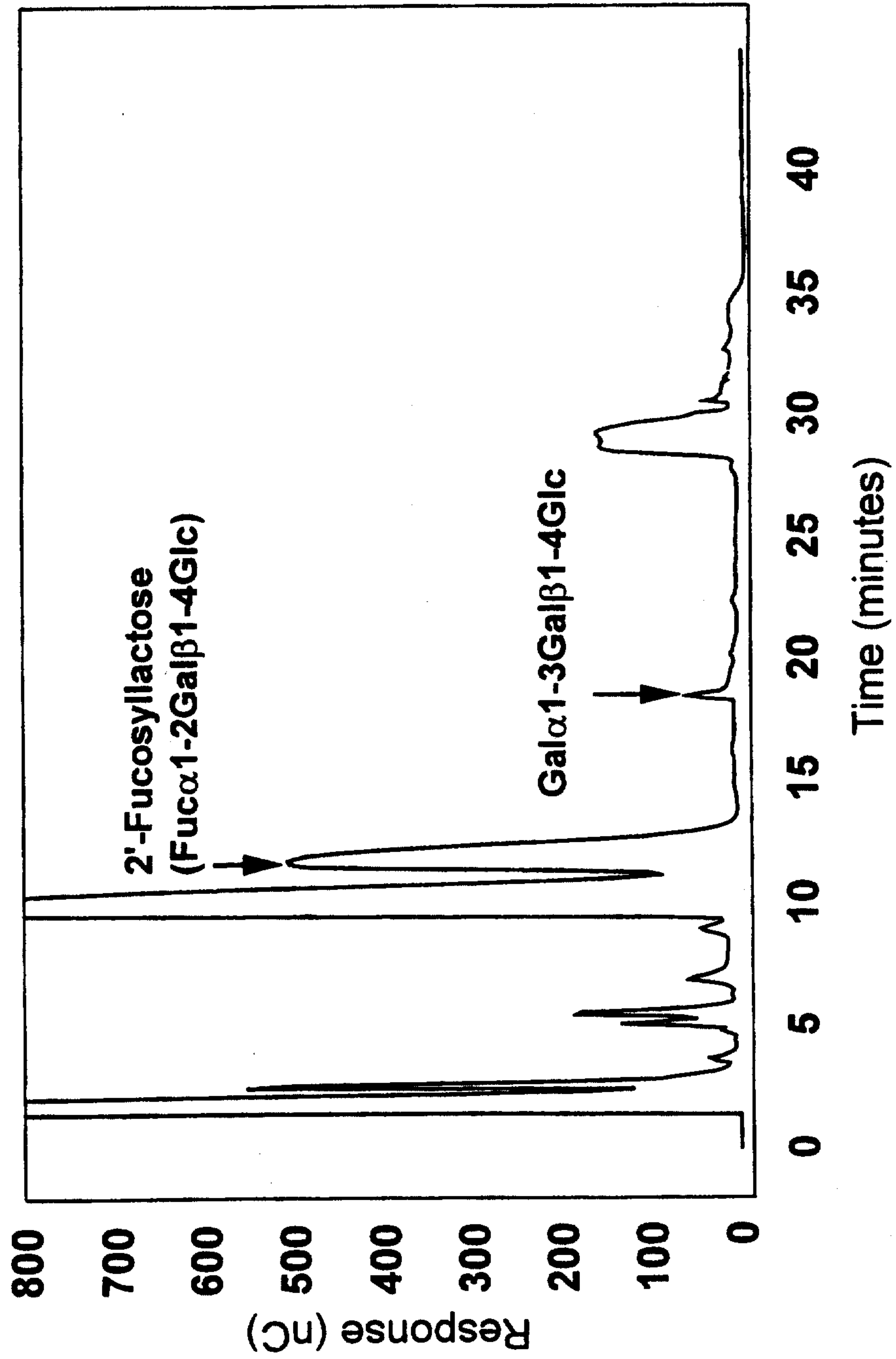


FIG. 18

24/26

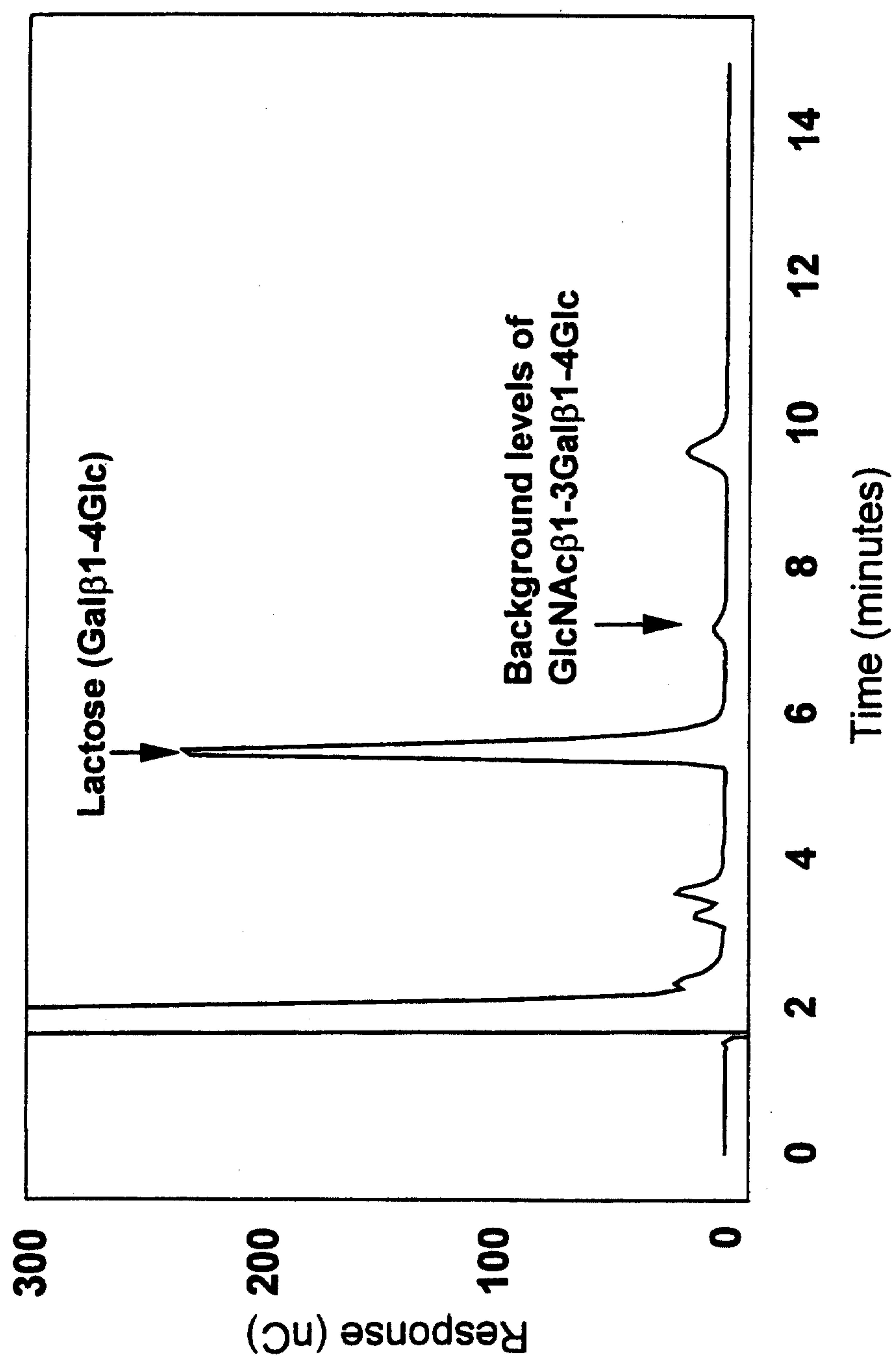


FIG. 19

25/26

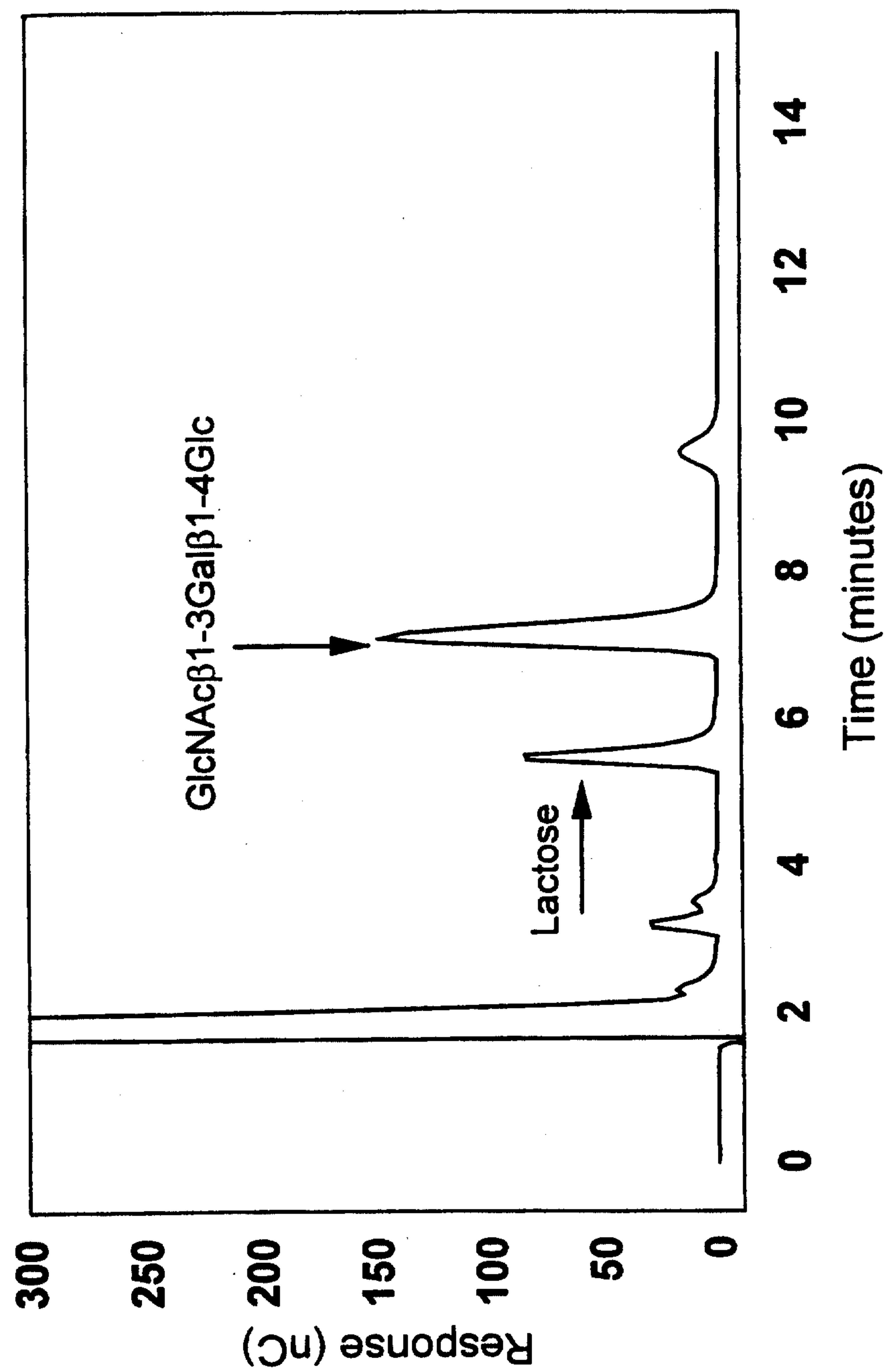


FIG. 20

26/26

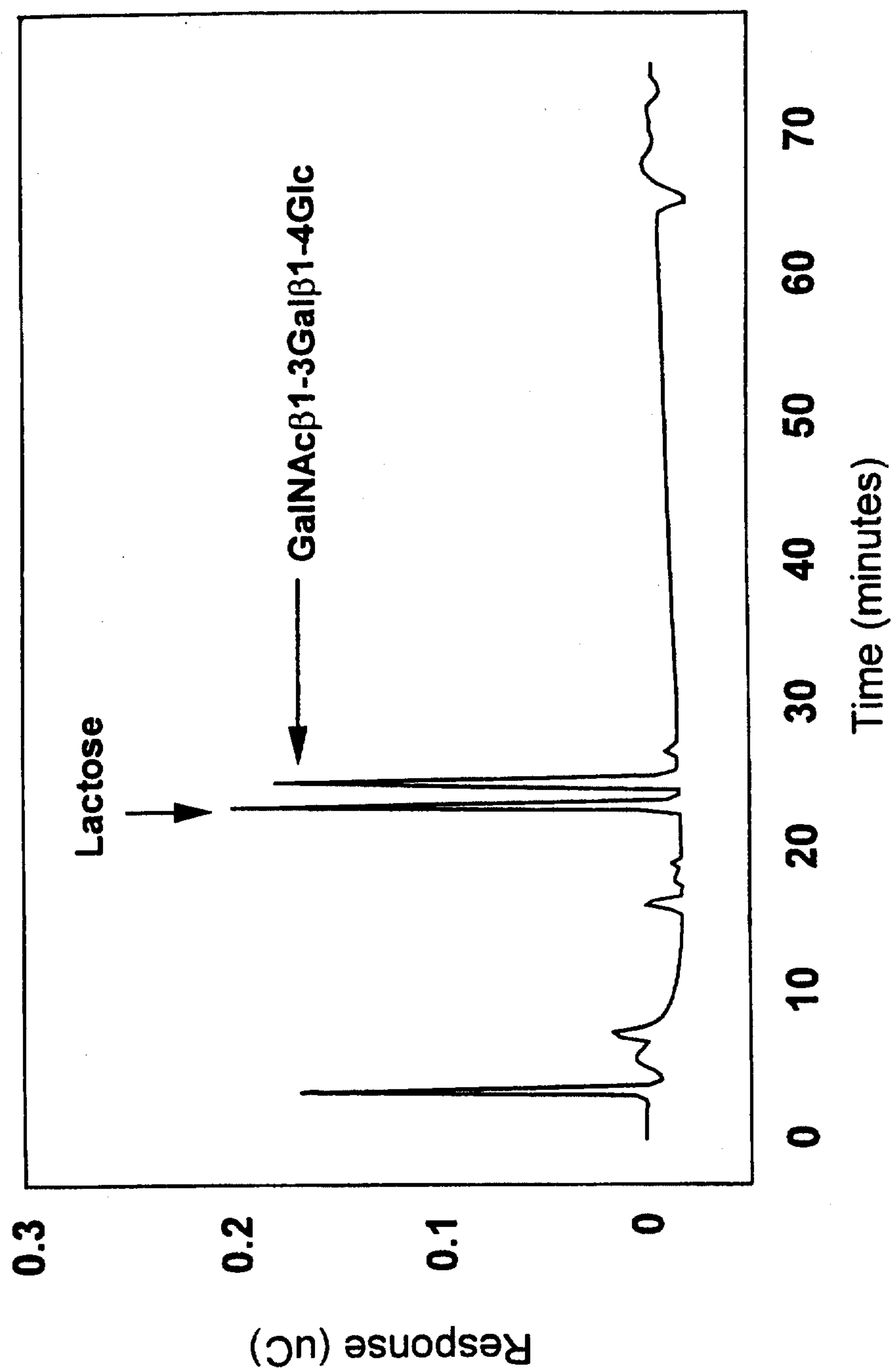


FIG. 21