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Improvements in the Process of Obtaining the Sialic Acid Donor

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Abstract: Ganglioside N-glicolyl GM_3 is over expressed in the tumour cells of breast cancer, while the Ganglioside N-acetyl GM_3 generally known to be associated to various cellular and membrane processes including the modulating of immune system, for these reason that in our country studies on the action of this gangliosides are made, to constitute a key element in obtaining the VSSP, which are used as adjuvant in therapeutic vaccines against renal cancer, prostate and immune activation in patients with HIV. Both gangliosides are investigated as fundamental components of therapeutic vaccines, and they are formed by a sialic acid unit (donor), a unit of lactose (acceptor) and a ceramide lipid moiety. The first is a key intermediate which is produced in three synthetic steps. This paper develops a modification to the current method of producing the sialic acid donor, where the trifluoroacetic acid was replaced by sulphuric acid in the reaction for obtaining the methyl ester, which reduces by half the reaction time. In the third stage, the purification by column chromatography is replaced by solid-liquid extraction.

Keywords: Ganglioside, N-acetyl GM_3, sialic acid donor, cancer.

1. Introduction

In 2013 an alternative treatment for cancer immunotherapy, which has been used significantly in recent years emerged. The immune response to tumours is mainly specific tumour antigens and does not damage healthy cells. Immunotherapy approaches applied are based on complementation, activation or stimulation of the immune system through a variety of compounds, including cytokines, vaccines, antibodies or effectors

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cells. The intention of cancer immunotherapy is to increase the weak host immune response to the tumour (active immunity) to combat the disease [1-3].

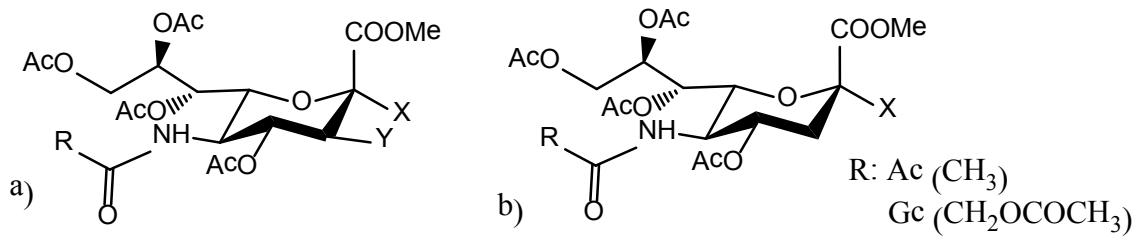
NGcGM3 ganglioside is over-expressed in tumour cells of breast cancer, and not found in the rest of human tissues, becomes under investigation for immunotherapy of this cancer. Similarly, it is known that the NGcGM3 ganglioside is used as component of adjuvant formulations who are studying disorders such as renal cancer, prostate cancer and immune activation in patients with HIV [2-4].

Obtaining these gangliosides can be natural or synthetic pathway. The first known that the yields are inadequate and there is a risk of introducing biological contaminants, and in the case of the synthetic pathway a pure product with defined structure is obtained [3].

Gangliosides are constituted by central unit lactose, a portion of ceramide and sialic acid unit, latter being the subject of study.

It is reported that donors sialic acid with an auxiliary group at C-3 (Y), possess good yields and high stereoselectivity in the reaction of sialylation with a variety of acceptors [5]. However, the preparation of these from sialic acid is laborious and then the removal of these auxiliary units require drastic conditions that can compromise the integrity of the molecule (Ph_3SnH, toluene, reflux, 10 h) [6].

Sialic acid derivatives with specific activation in the anomeric position (X), although they have all the negative structural features of sialic acid, have in their favour that are easy to prepare and then not require any further step in the synthesis to remove any substituent at C-3. For this, the substituent at the anomeric carbon should behave as good leaving groups, hence the most used are: halogen (Cl, Br) and thioglycosides (SPh, SMe) [5, 7-11]. For this reason, we can find as common substituents in glycosylation reactions of carbohydrates [12].



The synthesis of sialic acid donor consists of three stages: the first is the formation of sialic acid methyl ester, followed by replacing the hydroxyl group at the anomeric carbon by a chlorine atom. This substitution reaction is produced by first the formation of the peracetylated product, and then the anomeric acetate position is replaced by chlorine. The third stage involves obtaining thiophenyl, reaction occurs by transfer phase with

inversion of configuration. α-anomer is the product of interest. If you get something of the β- isomer, it is removed by leaching. The product obtained is the donor for the synthesis of N-acetyl GM_3. [10-11]

In this work, changes in the first and last stage of the process reporting performance improvements and reduction in total process time are realized. In the esterification reaction trifluoroacetic acid as catalyst is replaced by sulphuric acid at 10%. In the last purification step column chromatography process is removed and replaced by a process of solid-liquid extraction discontinued.

2. Experimental

2.1. Materials

Raw materials correspond to the steps that were evaluated in the process. N-acetyl neuraminic acid (98%, Carbosynth), dry methanol (0.003% H_2O, Merck), trifluoroacetic acid (for synthesis, Merck) and sulphuric acid (95 to 98%, Merck) as catalysts. Toluene, cyclohexane, ethyl acetate, and diethyl ether (Merck) as solvents, and silica gel 60 (0.2-0.5 mm) for chromatography (Merck).

2.2. Measurements

Nuclear Magnetic Resonance (NMR) are recorded at 0 and 25 °C on a Bruker Avance DPX / 250, using dimethylsulfoxide (DMSO-6D), chloroform ($CDCl_3$) and water (D_2O) deuterated solvents and tetramethylsilane (TMS) (δ = 0 ppm) as internal reference. The chemical shifts are expressed in δ scale (ppm). The multiplicity of the signals for protons is designated: singlet (s), doublet (d), doublet of double (dd), and multiplet (m). Synthesis processes are controlled by thin layer chromatography (TLC) using silica gel plates 60 F254 Merck. The solvent systems specified in each of the methods. The chromatoplates are revealed with H_2SO_4 solution in 5 % ethanol and heating at 105 °C for 5 minutes. The results obtained in the experimental design were processed using the program Statgraphics Centurion XV.

2.3. Obtaining of methyl (5-acetamido-3,5-dideoxy-α,β-D-glycero-D-galacto-2-nonulopiranosa) onato (methyl ester) using TFA

A mixture of 6 g of sialic acid, 120 mL of dry methanol was stirred about 5 minutes. Then, 1.2 mL of trifluoroacetic acid (TFA) is added dropwise and stirring continued at room temperature for 48 hours. The progress of the reaction is monitored by Thin Layer Chromatography (TLC), using chloroform: methanol (2:1; v/v) as solvent system and a solution of 5% H_2SO_4 in ethanol as a revelator. When the reaction is complete by chromatography then, the mixture is evaporated to dryness to give a white solid.

2.4. Obtaining of methyl (5-acetamido-3,5-dideoxy-α,β-D-glycero-D-galacto-2-nonulopiranosa) onato (methyl ester) using 10% H_2SO_4

0.42 mL of a 10 % solution of sulphuric acid in methanol is slowly added to 120 mL of dry methanol previously cooled between 0 and 5° C. Subsequently slowly added sialic acid and cooling is maintained for 5 minutes. Allowed to reach room temperature and stirring is continued for 24 hours more. The progress of the reaction is monitored by TLC, using chloroform: methanol (2:1; v/v) as solvent system and a solution of 5% H_2SO_4 in ethanol as a revelator. Finished the reaction (by chromatography) the mixture was evaporated to dryness by rotaevaporation and co-evaporated five times with 30 mL of toluene to dryness, obtaining a white solid.

2.5. Semipurification of thiophenyl by solid-liquid extractions

Thiophenyl crude is completely dissolved in dichloromethane. Silica gel 60 (0.2-0.5 mm) is added and evaporated for impregnating the product to constant weight. Subsequently, it is extracted firstly with cyclohexane: ethyl acetate (5: 1; v/v) in order to remove higher Rf impurities or less polar and then, extracted with ethyl acetate to elute the product of interest. This operation is performed with mechanical stirring for periods of 30 minutes at room temperature. The extractions are monitored by TLC, using as solvent system toluene: acetone (2:1; v/v) and as developer a solution of 5% H_2SO_4 in ethanol. The final product has an appearance of sparkling white solid.

2.6. Final purification of thiophenyl with diethyl ether

Finally, the semi-purified product is treated by leaching with adding of 3 mL of diethyl ether per gram of product, stirred 30 minutes at room temperature with magnetic stirring and after this time, it is kept cold (2-8 °C) for 1 hour. Then, filtered and washed with cold diethyl ether, dried under vacuum at room temperature.

2.7. Determining operating conditions in the stage of solid-liquid extraction using an experimental design

In order to study the effect of varying amount of silica gel and volume of solvent to be employed in the purification step of crude thiophenyl, a design of experiment 3^2 was performed with two replicates at the centre point.

Process conditions:

m: mass of thiophenyl crude

Extraction temperature: room temperature

Volume of dichloromethane to dissolve the thiophenyl crude: 19.4 x m

Extraction time: 30 minutes

Solvent to remove impurities: Cyclohexane: ethyl acetate (5:1; v/v)

Solvent to extract the product: Ethyl acetate

Extraction variables:

1. Amount of solvent for the extraction: three levels are considered: (19.4 x m), (24.3 x m) and (29.1 x m).
2. Amount of Silica gel to impregnate: Considered three levels: 4 x m, 7 x m and 10 x m.

Table 2.1 Factorial design to determine the influence of the amount of silica and volume of solvent to be used in solid-liquid extractions.

Trials	A	Silica gel (g)		B	Volume (mL)	
1	-1	4 x m	12.36	-1	19.4 x m	60
2	0	7 x m	21.63	-1	19.4 x m	60
3	+1	10 x m	30.9	-1	19.4 x m	60
4	-1	4 x m	12.36	0	24.3 x m	75
5	0	7 x m	21.63	0	24.3 x m	75
6	+1	10 x m	30.9	0	24.3 x m	75
7	-1	4 x m	12.36	+1	29.1 x m	90
8	0	7 x m	21.63	+1	29.1 x m	90
9	+1	10 x m	30.9	+1	29.1 x m	90
10	0	7 x m	21.63	0	24.3 x m	75
11	0	7 x m	21.63	0	24.3 x m	75

Each experiment was performed with 3.09 g of crude oil thiophenyl obtained under the conditions described in section 2.5, five extractions were performed with cyclohexane: ethyl acetate (5:1; v/v) to remove impurities and four extractions with ethyl acetate to extract the main product.

2.8. Obtaining process sialic acid donor from a higher volume

This stage was carried out from 20 g of sialic acid with better working conditions specified in the Experimental Design. So 1L reactors were used for the first and second stage, 3L for the third stage and at the stage of solid-liquid extraction was used a 6 L reactor with filter plate and mechanical agitation.

3. Results and Discussion

3.1 Obtaining of methyl (5-acetamido-3,5-dideoxy-α,β-D-glycero-D-galacto-2-nonulopiranosa) onato (methyl ester)

It is reported, the first reaction of the sialic acid donor [10-11] corresponding to ester formation use of trifluoroacetic acid as catalyst in methanol solvent. It is also known that sulphuric acid can be used as a catalyst, and we believe this assessment to be less expensive [13].

To do this, different molar ratios ranging from 0.0026 to 0.00032 moles were studied. These are shown in Table 3.1 and the form of acid addition too. It can be seen that when low molar ratios of concentrated sulphuric acid is used a signal around δ 3.13 ppm (Figure 3.1) that does not appear in the reference 1H NMR spectrum of methyl ester was observed.

Table 3.1. Experiments to determine the necessary amount of concentrated H_2SO_4 in the Process.

Exp.	**Reactive**	**Amount**		**Moles**	Observations
		g	**mL**		
1	TFA	0.296	0.2	0.0026	----
2	H_2SO_4	0.1275	0.069	0.0026	----
3	H_2SO_4	0.1275	0.069	0.0026	Change in the addition of reagents
4	H_2SO_4	0.0635	0.0345	0.0013	Change in the addition of reagents
5	H_2SO_4	0.0317	0.01725	0.00064	----

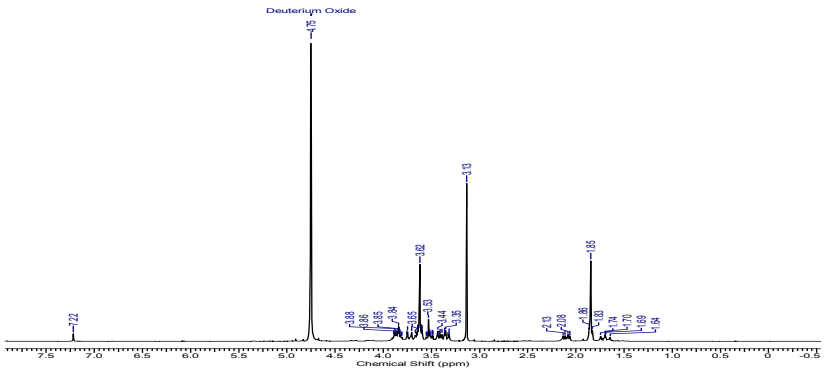


Figure 3.1. Spectrum corresponding to obtain the methyl ester with concentrated H_2SO_4

Considering the strength of the acid it was decided to prepare a 10% solution of sulphuric acid in dry methanol and change the order of addition. This allows us to observe by TLC (Figure 3.2) a single spot, showing in 1H NMR spectrum (Figure 3.1) the same signals corresponding to the reference spectrum product (Figure 3.3).

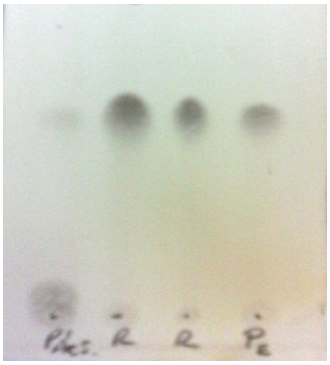


Figure 3.2. Thin layer chromatography corresponding to reaction with H_2SO_4 at 10% [Pp: Starting raw material in reaction (N-acetyl neuraminic Acid)], R: Reaction of the methyl ester obtained by the use of 10% H_2SO_4, P_E: Reference substance of methyl ester).

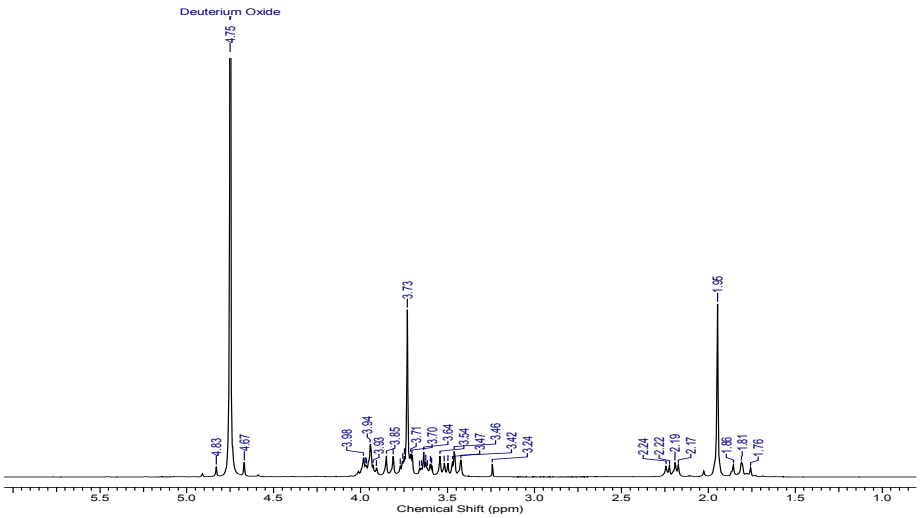


Figure 3.3. NMR ^{1}H spectrum corresponding reference reaction to obtain methyl ester.

Treating of reaction was carried out by performing successive coevaporations with toluene which can remove dimethyl sulphate that may have formed as a by-product thereof.

In view of reproduce the results, three replicates of the process were performed using the current method (TFA), and modified method (H_2SO_4 at 10%), starting from 6 g of sialic acid. The results are shown in Table 3.2.

Table 3.2. Results obtained in the reaction of the methyl ester using TFA and H_2SO_4 at 10%

Acid	**Parameters to be evaluated**	**Test**		
		1	**2**	**3**
TFA	m(g)	6.4	6.3	6.3
	Time (h)	48	48	48
	% Performance	106.7	105	105
10 % H_2SO_4	m(g)	6.3	6.3	6.4
	Time (h)	23	25	24
	% Performance	105	105	106.7

As can be seen in Table 3.2, there is ~~a~~ considerable reduction in reaction time when using H_2SO_4 at 10%, without affecting the performance thereof. To compare the results obtained with the use of H_2SO_4 at 10% and TFA, Statgraphics Centurion XV is used; the box and whisker graphic obtained and shown in Figure 3.4.

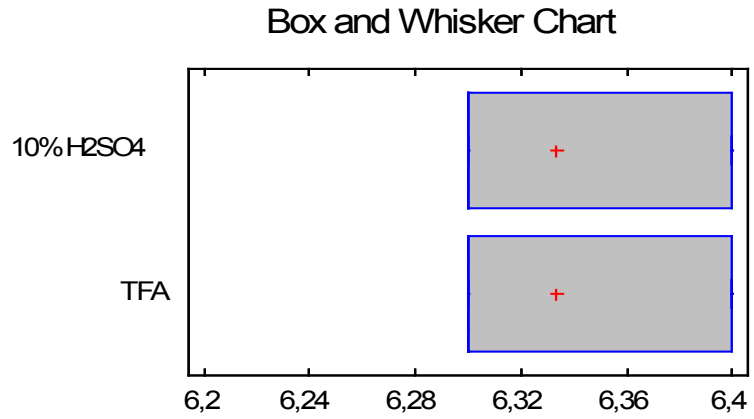


Figure 3.4. Box and whisker plot to compare two samples with the use of H_2SO_4 at 10% and TFA.

The graph of Figure 3.4 shows that both the use of TFA as the H_2SO_4 at 10% equal amounts of methyl ester are obtained, with the advantage of using sulphuric acid the reaction time is reduced of 48 to 24 hours. These results lead to energy savings and increased productivity, reason why its use is more efficient in the process to obtain methyl ester.

3.2 Replacement of chromatographic purification

In the current process of thiophenyl crude (third stage of the process) is purified by column chromatography to remove impurities. In this procedure the crude product was passed through a column with the use of ethyl acetate as the only solvent, so that a large number of fractions appear mixed with impurities. Moreover, the process is limited in increasing scale. Therefore it was decided to study the purification with the use of a batch process.

The thiophenyl crude is characterized by having more non polar impurities of the product of interest. For this reason, it is decided to remove these with a cyclohexane: ethyl acetate (5:1; v/v) and then extracted the major product with ethyl acetate (AcOEt).

The experimental design conditions employed is described in the experimental part. In Table 3.3 the obtained results therein for removing impurities by solid-liquid extractions are shown. To determine the effect of volume mixture of cyclohexane: ethyl acetate (5:1; v/v) and the amount of silica gel used on the total amount of impurities removed, the Statgraphics Centurion XV is used. The results are shown in the Pareto chart, Figure 3.5.

Table 3.3. Results obtained in the solid-liquid extractions using cyclohexane: AcOEt (5:1; v/v) to remove impurities

Silica gel (g)	**Volume (mL)**	Weight impurities **(g)**				
		∑ Impurities **(g)**				
		1	**2**	**3**	**4**	**5**
12.36	60	0.54	0.126	0.035	0.028	0.035
		0.54	0.666	0.701	0.729	0.764
12.36	75	0.523	0.125	0.055	0.03	0.026
		0.523	0.648	0.703	0.733	0.759
12.36	90	0.569	0.141	0.03	0.035	0.039
		0.569	0.71	0.74	0.775	0.814
21.63	60	0.499	0.122	0	0.01	0.012
		0.499	0.621	0.621	0.631	0.643
21.63	75	0.510	0.099	0.030	0.016	0.014
		0.510	0.609	0.639	0.655	0.669
21.63	90	0.496	0.116	0.027	0.022	0.017
		0.496	0.612	0.639	0.661	0.678
30.9	60	0.468	0.148	0.016	0	0
		0.468	0.616	0.632	0.632	0.632
30.9	75	0.514	0.084	0.025	0.010	0.007
		0.514	0.598	0.623	0.633	0.64
30.9	90	0.46	0.118	0.046	0.012	0.002
		0.46	0.578	0.624	0.636	0.638
21.63	75	0.475	0.123	0.034	0.012	0.013
		0.475	0.598	0.632	0.644	0.657
21.63	75	0.48	0.117	0.014	0.015	0.011
		0.48	0.597	0.611	0.626	0.637

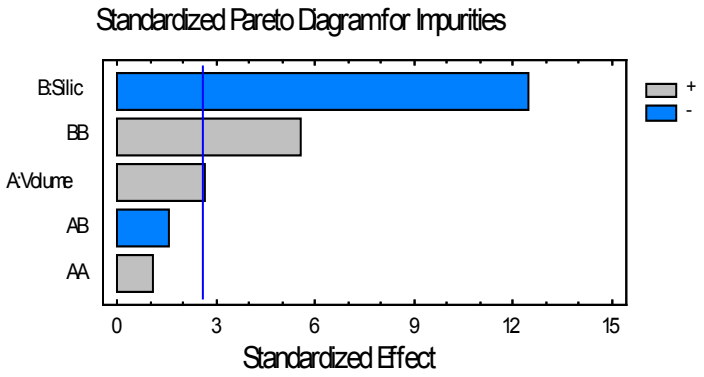


Figure 3.5. Significance volume mixture of solvents and the amount of silica gel on the mass of impurities extracted

Statistical analysis allows us to select the amount of silica gel to be used. This proves to be significant with negative effect, whereas the volume of the mixture of cyclohexane: ethyl acetate is significant with positive effect. This means that with the least amount of silica gel and the largest volume of solvent mixture extracted mass impurities increases. Moreover, the interaction of both variables was not statistically significant. From polynomial adjusted for the effect of the variables on the total mass of impurities obtained in the extraction, Statgraphics Centurion XV gave the response surface as shown in Figure 3.6.

Mass of impurities extracted = 1.02487 + 0.00101111 * Volume - 0.0333681 * Silica gel +0.000593874 * Silica gel^2

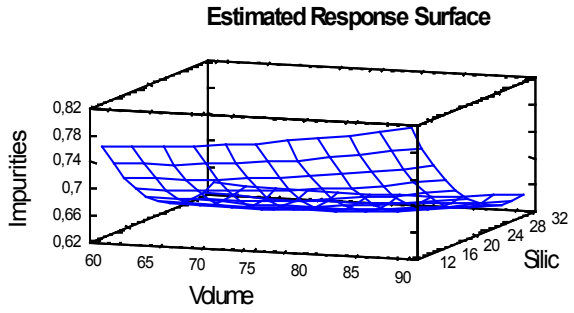


Figure 3.6. Response surface of the mass of impurities extracted

The corresponding fraction of the non polar impurities obtained is characterized by ^{1}H and ^{13}C NMR and as a result it was demonstrated that corresponded to the potassium salt of unreacted thiophenol (Figure 3.7).

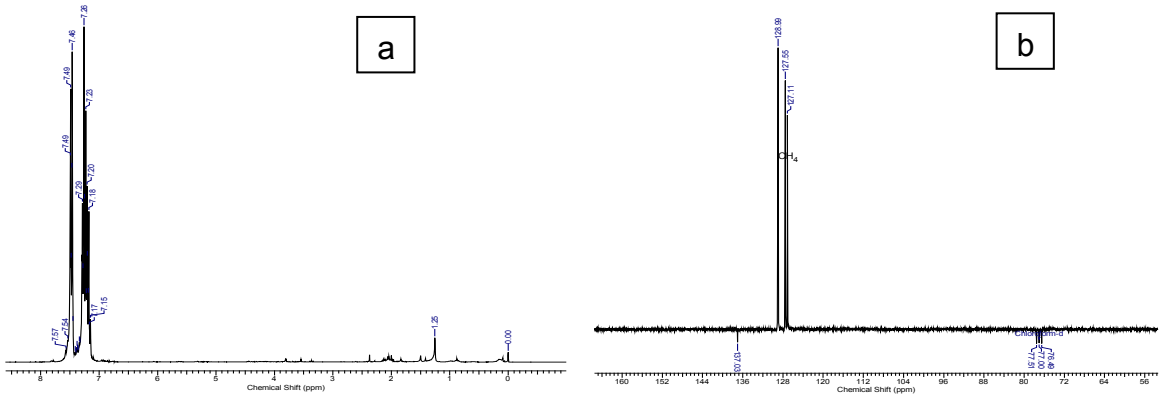


Figure 3.7. ^{1}H (a) and ^{13}C (b) NMR made with impurities isolated in the solid-liquid extractions the thiophenyl crude (potassium salt of thiophenol)

For the extractions of the semi-purified (sialic acid donor) ethyl acetate is used as sole solvent. The result of the experimental design is shown in Table 3.4, where a total of four extractions are performed to determine which parameters are significant.

Table 3.4. Results obtained in the solid-liquid extractions using AcOEt for elution of sialic acid donor

Silica gel (g)	**Volume (mL)**	**Product Weight (g)**			
		∑ Product (g)			
		1	**2**	**3**	**4**
12.36	60	1.327	0.506	0.192	0.096
		1.327	1.833	2.025	2.121
12.36	75	1.269	0.507	0.214	0.073
		1.269	1.776	1.99	2.063
12.36	90	1.422	0.439	0.165	0.061
		1.422	1.861	2.026	2.087
21.63	60	0.887	0.655	0.330	0.161
		0.887	1.542	1.872	2.033
21.63	75	1.057	0.592	0.276	0.178
		1.057	1.649	1.925	2.103
21.63	90	1.077	0.595	0.313	0.153
		1.077	1.672	1.985	2.138
30.9	60	0.581	0.661	0.364	0.145

		0.581	1.242	1.606	1.751
30.9	75	0.712	0.665	0.379	0.229
		0.712	1.377	1.756	1.985
30.9	90	0.921	0.584	0.320	0.205
		0.921	1.505	1.825	2.03
21.63	75	0.939	0.651	0.320	0.163
		0.939	1.59	1.91	2.073
21.63	75	1.075	0.568	0.296	0.170
		1.075	1.643	1.939	2.109

Also the effect of the volume of ethyl acetate and the amount of silica gel used on the total amount of extracted product was processed in the Statgraphics Centurion XV. In Figure 3.8 the resulting Pareto chart is showed.

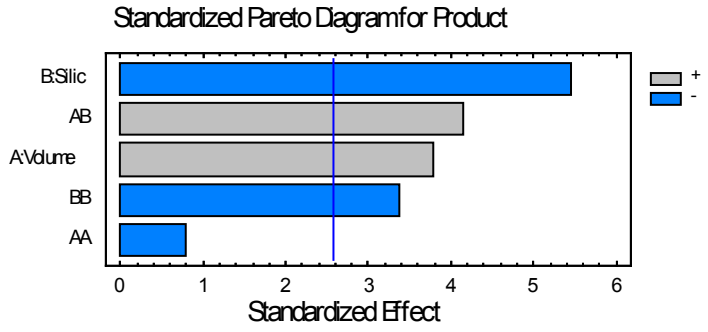


Figure 3.8. Volume of AcOEt significance and the amount of silica gel on the extracted product mass

Statistical analysis shows significant results as the amount of silica gel and interaction with itself (both negative effect) as well as the volume of ethyl acetate and its interaction with silica gel (both positive effect). This implies that with a lower amount of silica gel and higher volume of ethyl acetate increased the mass of extracted product is produced. By using the Statgraphics Centurion XV, the adjusted polynomial for the effect of the variables is obtained as well as the response surface graph shown in Figure 3.9.

Adjusted polynomial:

Product mass extracted = 2.44588 – 0.00828333*Volume – 0.00847837*Silica gel + 0.000562747*Volume*Silica gel – 0.000989532*Silica gel ^2

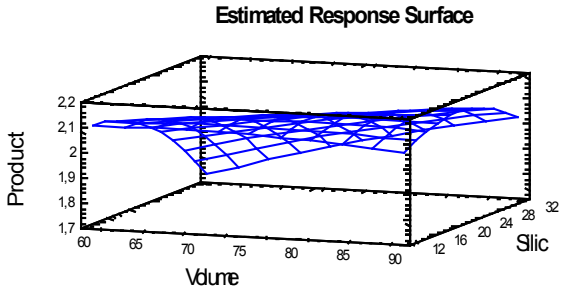


Figure 3.9. Response surface of the product mass extracted.

From the results, we can conclude that both the removal of impurities like so fundamental product, the best operating conditions to achieve the best yields are achieved working with 12.36 g of silica gel (4 x m) and 90 mL of solvent (29.1 x m).

The maximum number of extractions to make impurities in the working conditions must be four, shown with the table 3.5 data and the depletion curve of Figure 3.10.

Table 3.5. Results obtained in the extraction of the impurities to selected working conditions

Silica gel **(g)**	**Thiophenyl crude (g)**	**Volume (mL)**	**Weight impurities (g)**				
			∑ impurities (g)				
			1	**2**	**3**	**4**	**5**
12.36	3.09	90	0.569	0.141	0.03	0.035	0.039
			0.569	0.71	0.74	0.775	0.814

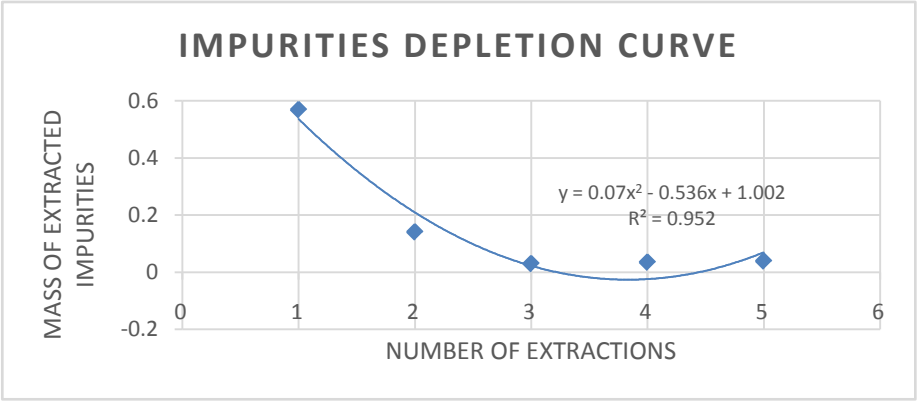


Figure 3.10. Depletion curve impurities

Moreover, the maximum number of extractions to make main product in the working conditions must be six, shown with the table 3.6 data and the depletion curve of Figure 3.11.

Table 3.6. Results obtained in the extraction of the product for the job conditions selected (12.36 g of silica gel and 90 mL of solvent)

Product Weight (g)							
∑ Product (g)							
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1.422	0.439	0.165	0.061	0.028	0.015	0.010	0.002
1.422	1.861	2.026	2.087	2.115	2.13	2.14	2.142

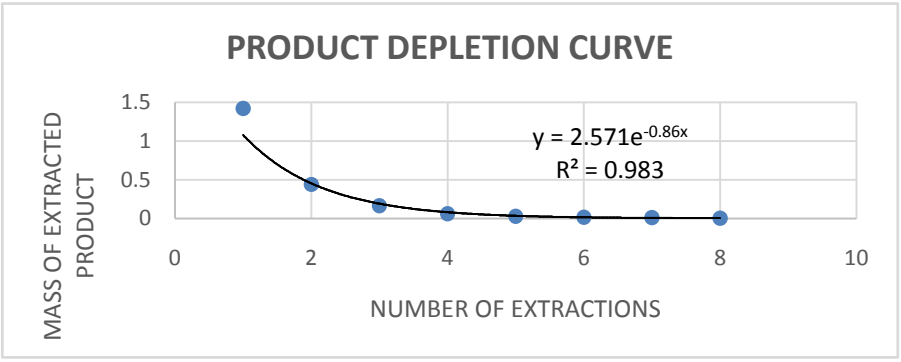


Figure 3.11. Product depletion curve

In general, the results of experimental design demonstrated that can obtain the sialic acid donor semipurified using solid-liquid extraction with a minor amount of silica gel (4 x m) and the largest volume (29.1 x m).

Once obtained semi purified sialic acid donor, it is needed to remove the β-anomer (higher Rf) that impurities the product. This is done through a process of leaching with diethyl ether.

The yields of the current column process are about 51% and using the treatment proposed in this paper reaches 58%, which exceeds the reported value.

The final pure product was analyzed for purity versus a reference ^{1}H NMR spectrum (Fig.3.12a and 3.12b). The perfect match is shown in most of the signals of the two spectra.

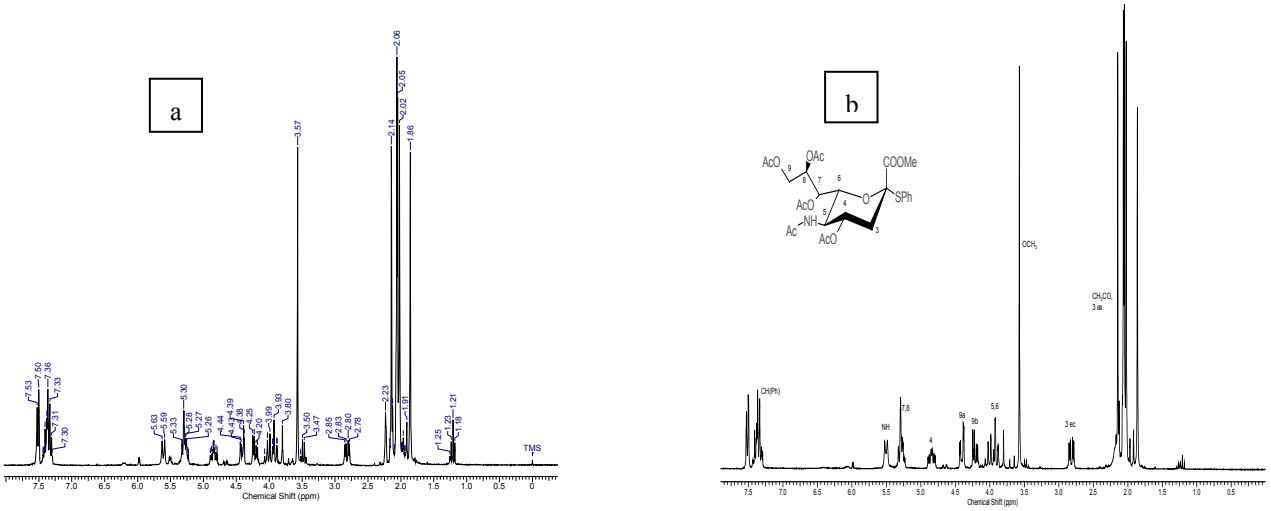


Figure 3.12. ^{1}H NMR performed to the obtained product (a) and reference spectrum of sialic acid donor (b)

The spectrum performed shows two signals as multiplets at δ 7.3 and 7.5 ppm corresponding to the thiophenyl group and two signal shaped singlet at δ 1.8 and 2.23 ppm corresponding to the protons of methyl group for acetate groups. Next, it is observed signal shaped singlet at δ 3.6 ppm, associated to the protons of the methyl group of the OCH_3 group, besides being the rest of the protons signals of the reference spectrum, allowing confirming the purity of the product.

4. Conclusions

It demonstrated that replacing the trifluoroacetic acid by sulphuric acid for the esterification step leads to lower process time.

It was shown that replacing the column chromatographic process for discontinued extractions leads to a higher yield with less use of the amount of silica gel.

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