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A SIMPLE METHOD FOR THE SIMULTANEOUS EXTRACTION OF CHOLESTEROL AND PHOSPHATIDES FROM BOVINE SPINAL CORD WITH PARTICULAR REFERENCE TO PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE

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A large-scale process is described for the simultaneous extraction of cholesterol, phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) from bovine spinal cord. The process is essentially of batch type and consists of four stages, each requiring a different solvent.

Total lipids are first extracted from the minced and dried spinal cord with *n*-hexane. The concentrated extract is then stirred with *n*-hexane: acetone mixture (10:90 v/v) to separate cholesterol from the phosphatides which are filtered in the Sparkler's filter under inert atmosphere. Cholesterol is recovered from the acetone extract by distillation and crystallisation from methanol. Phosphatides are extracted with ethanol to selectively remove PC and PE by recycling ethanol through the Sparkler's filter. The ethanol extract is chilled overnight and the precipitated PE filtered off. PC is recovered by distilling the solvent under vacuum. The recovery of cholesterol PC and PE on dry weight basis is 12, 10 and 1% respectively.

The process is comparatively simple and economically feasible for a large-scale production of the title products.

INTRODUCTION

Various methods have been described in the literature for the large-scale extraction of lipids from spinal cord and other nerve tissue [1, 2, 3, 4] which deal mainly with extraction of cholesterol. However, in addition to cholesterol, the spinal cord contains about 60% phosphatides which are also important commercially. In view of this, simultaneous extraction of these products is desirable to increase the viability of the process for commercial exploitation.

In a previous study [5] we had described a method for the simultaneous extraction of cholesterol and phosphatides. In this communication we describe a method for the extraction of cholesterol and the separation of phosphatides into individual products. Since the phosphatides are a very complex mixture and require diverse methods for their resolution into individual components, effort have been concentrated on recovering PC and PE only which are comparatively easy to isolate from the rest of phosphatides.

EXPERIMENTAL

Spinal cord was removed from the slaughtered bovine carcasses and collected in drums containing preservative (5% formalin wt/vol) as described earlier [5]. In the laboratory the material was desheathed, minced and dried before being subjected to extraction procedure.

One kg dried spinal cord was extracted in a continuous Soxhelet apparatus with about 12 litres of *n*-hexane until the lipids were completely extracted (7 to 8 cycles) as shown by the disappearance of colour from the raw material. The extract so obtained was siphoned into the solvent recovery unit fitted with a stirrer and condenser (flow diagram). Here the extract was heated and stirred and the solvent distilled off until about 1 to 1.5 litre of the solvent was left. The concentrated hot extract was then sucked below into the extractor vessel fitted with a stirrer, motor and a gear box and containing about 7 litres of acetone. The mixed contents were stirred for about 10 min. so that the cholesterol was dissolved selectively in the acetone phase and the phosphatides separated. The contents were then pumped through a Sparkler's filter unit where phosphatides were retained after filtration and the clear extract of cholesterol in acetone was collected at the end of the line. Throughout the process, inert atmosphere was maintained by passing a slow stream of nitrogen through the whole unit. About 4 litres of ethanol was added to the extractor unit and pumped through the Sparkler's filter. The solvent was recycled 7 or 8 times so that PC and PE were selective-

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ly recovered from the phosphatide mass and collected at the end of the line. Residual phosphatides were removed after opening the lid of the Sparkler's filter and collected separately. The extract containing PC and PE was chilled overnight at about 8 to 10°C. The crude PE that settled down at this temperature was collected by cold filteration. The solution containing crude PC was subjected to distillation in a unit fitted with a suction pump assembly, the last traces being removed under vacuum. The dried product was collected and stored under a nitrogen atmosphere.

The acetone extract containing crude cholesterol was also subjected to distillation. Cold water was cycled through the condenser to minimise solvent losses. Last traces of the solvent were removed under vacuum. The dried crude cholesterol was dissolved in 2.5 litres of methanol, concentrated to about 1 litre and then left overnight for crystallisation. The crystals were collected by filtraction and the mother liquor was distilled to recover the solvent. An oily fraction which contains impurities was left behind together with some leftover cholesterol and phosphatides.

The unextractable matter left behind after the recovery of lipids consists of neurokeratin network and forms the 'skeleton' of the spinal cord. This fraction was subjected to a pressure of 10 lb p.s.i. to recover most of the solvent absorbed in it.

RESULTS AND DISCUSSION

Flow diagram given below shows the arrangement of the units used and an outline of the steps involved in the process.

FLOW DIAGRAM

Data regarding the recovery of various products are given in Table 1.

Flow diagram
Extraction of cholesterol and phospholipids (Lecithin & cephalin) from bovine spinal cord

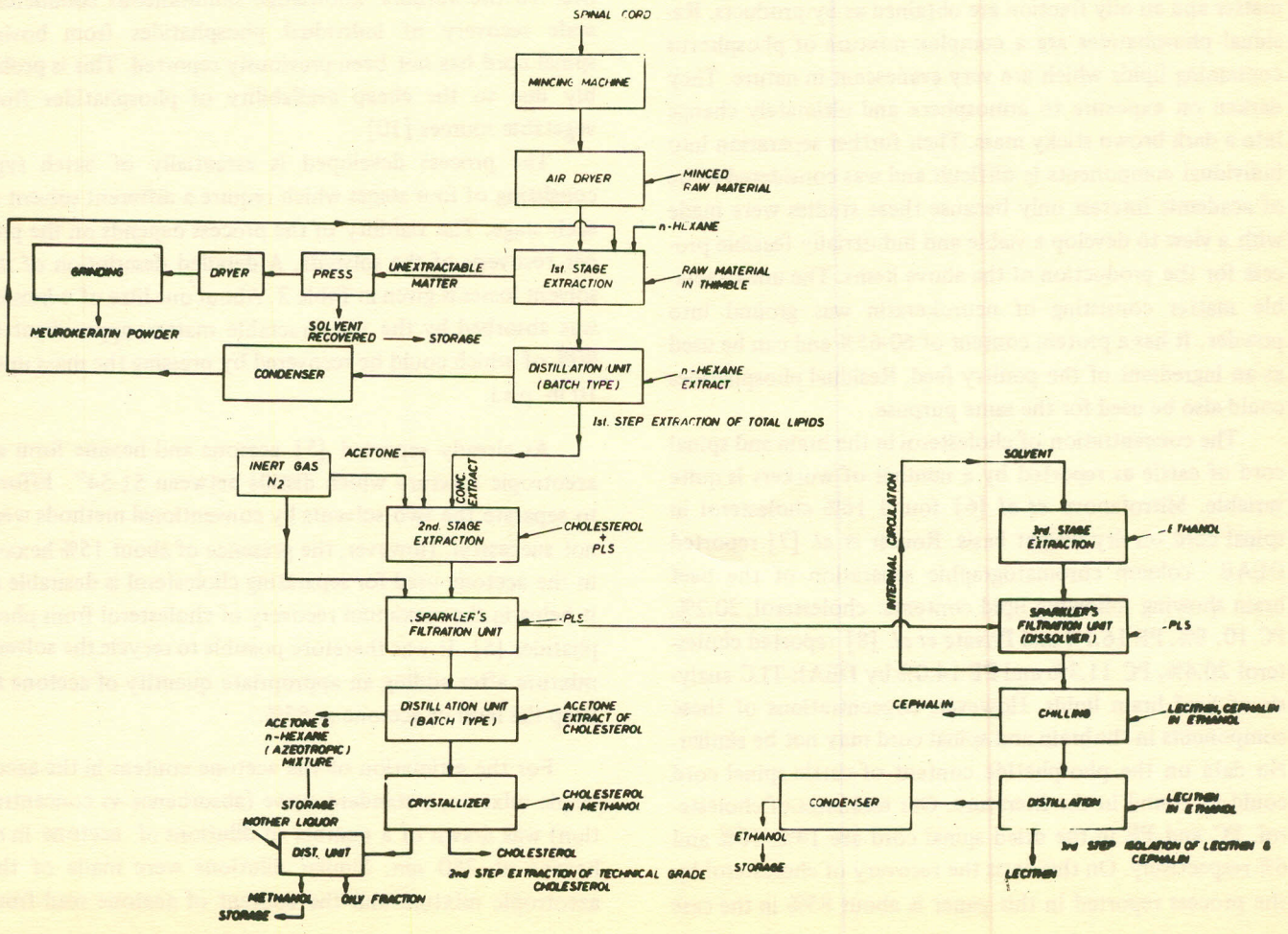

Table 1.

Product recovered	Yield (on dry weight basis)
Cholesterol (Technical Grade) (On purification yields pharmacopoeal grade product-10%)	12%
Phosphatidyl Choline (PC) (Commercial Grade)	9 – 10%
Phosphatidyl ethanolamine (PE) (Commercial Grade)	1%
By-products: Neurokeratin (unextractable matter consisting of 60-65% protein)	30 – 32%
Residual phosphatides	28 – 30%
Others (oily liquid)	17 – 20%

Spinal cord is totally wasted after the slaughter of the animal. The aim of developing the present method was to process it in such a manner that it could be fully exploited commercially. After extracting cholesterol, PC and PE which are the main products, residual phosphatides, unextractable matter and an oily fraction are obtained as by-products. Residual phosphatides are a complex mixture of phosphorus containing lipids which are very evanescent in nature. They darken on exposure to atmosphere and ultimately change into a dark brown sticky mass. Their further separation into individual components is difficult and was considered to be of academic interest only because these studies were made with a view to develop a viable and industrially feasible process for the production of the above items. The unextractable matter consisting of neurokeratin was ground into powder. It has a protein content of 60-65% and can be used as an ingredient of the poultry feed. Residual phosphatides could also be used for the same purpose.

The concentration of cholesterol in the brain and spinal cord of cattle as reported by a number of workers is quite variable. Mitrofanova *et al* [6] found 16% cholesterol in spinal cord on dry weight basis. Rouser *et al.* [7] reported DEAE column chromatographic separation of the beef brain showing following lipid contents: cholesterol, 20.2%, PC 10. 8%, PE 16.1% and Private *et al.* [8] reported cholesterol 20.4%, PC 11.3% and PE 14.0% by DEAE-TLC analysis of beef brain lipids. However, concentrations of these components in the brain and spinal cord may not be similar. No data on the phosphatide content of cattle spinal cord could be found in the literature. Our estimates of cholesterol, PC and PE in the dried spinal cord are 14%, 10% and 6% respectively. On this basis the recovery of cholesterol by the process reported in this paper is about 85% in the case of cholesterol and 90% in the case of PC. The recovery of PE however is low. In one experimental trial known quantities of cholesterol, PC and PE (50 g, 25 g, and 10 g) respectively) were added to the minced spinal cord and subjected to extraction procedure as usual. A more or less corresponding increase in the yield of the products was obtained.

The process for the simultaneous recovery of cholesterol and phosphatides described here is comparatively simple. After extraction of total lipids with *n*-hexane, cholesterol is extracted with acetone: hexane mixture and phosphatides are filtered in the Sparkler's filter from which PC and PE are selectively extracted by ethanol. Shiffered and Porsche [1] obtined about 9% cholesterol from the dried spinal cord by extraction with dichlorethane, the phosphatides being precipitated by addition of water but the details of phsphatide recovery from the aqueous phase have not been described. Choesterol was also obtained by the countercurrent extraction of the animal tissue with organic solvents in 72 to 80% yield [9]. Kovatsits *et al* [2] obtained 6.5% cholesterol from the cattle spinal cord using dichloroethane as a solvent but did not recover phosphatides. To the authors knowledge simultaneous commercial scale recovery of individual phosphatides from bovine spinal cord has not been previously reported. This is probably due to the cheap availability of phosphatides from vegetable sources [10].

The process developed is essentially of batch type consisting of four stages which require a different solvent at each stage. The viability of the process depends on the proper recovery of the solvents. A detailed description of the solvent losses is given in Table 2. About one litre of *n-hexane* was absorbed by the unextractable matter, approximately 80% of which could be recovered by pressing the mass upto 10 lb. p.s.i.

As already reported [5] acetone and hexane form an azeotropic mixture which distills between 51-54°. Efforts to separate the two solvents by conventional methods were not successful. However, the presence of about 15% hexane in the acetone used for separating cholesterol is desirable as it helps in the maximum recovery of cholesterol from phosphatides [5]. It was therefore possible to recycle the solvent mixture after adding an appropriate quantity of acetone to keep the level of acetone at 85%.

For the estimation of the acetone content in the azeotropic mixture, a standard curve (absorbence vs concentration) was drawn of a number of dilutions of acetone in *n*-hexane at 280 nm. Similar dilutions were made of the azeotropic mixture and the content of acetone read from

Table 2. Solvent losses at various stages of extraction

Solvent used	Stage of the process	Loss in litres per kg. dried spinal cord
First stage (*n*-hexane)	*Extraction of total lipids*	
	(a) With unextractable matter	0.985 litre
	(b) With concentrated extract	0.076 litre
	(c) General processing losses	0.152 litre
	Total:-	1.213 litres
Second Stage (acetone)	*Separation of cholesterol*	
	(a) With the solid residue	0.450 litre
	(b) Retained in the equipment and processing losses	0.512 litre
	(c) Replenishing amount to keep the concentration at 85%	1.410 litres
	Total:-	2.372 litres
Third stage (ethanol)	*Recovery of PC & PE*	
	(a) Retained in the filter and line	0.252 litre
	(b) Retained in the solids and other processing losses	0.250 litre
	Total:-	2.372 litres
Fourth stage (methanol)	*Crystallisation of cholesterol*	
	(a) Absorbed or associated with the crystals	0.195 litre
	(b) Retained with the fatty matter and other processing losses	0.130 litre
	(c) Evaporation losses during purification	0.325 litre
	Total:-	0.650 litre

the standard curve.

The method used for the recovery of commercial grade PC and PE was based on their relative solubilities in ethanol. Both are soluble in ethanol, but a major portion of PE was found to precipitate on chilling overnight. PC remains in solution and can be recovered by distilling the ethanol. Separation of PC and PE could also be achieved by making cadmium chloride addition complexes [11] the complex with PC being much less soluble than the complex with PE, can be separated. However, this method is complicated and would have added to the cost of the process and therefore the simpler method referred to above was adopted for the recovery of commercial grades of PC and PE. Further purification into comparatively purer products could be achieved by treatment with cadmium chloride (II) or magnesium sulphate if so desired [12].

The so-called commercial PC which is used for general purposes is obtained from the soybean as a by-product in the manufacture of soybean oil. For this reason it is quite cheap and easily available, but it contains total phosphatides of the soybean in addition to 30-40% oil. Egg PC is very costly in view of the costly raw material from which it is recovered but is of superior quality and is used for pharmaceutical purposes. PC from the spinal cord could be a good substitute of egg PC and would be comparatively cheaper if produced as a by-product of cholesterol manufacture from spinal cord.

REFERENCES

1. R.H. Shiffered and J.D. Porsche, US Pat. 2, 494, 726, Jan. 17, 1950.
2. M. Kovatsits, J. Gyurko and I. Lengeyel, Acta. Farm. Hung., 31 Suppl., 102 (1961).
3. H. Frantisec, C. Jindrich, C. Josef and N. Irena, Czech pat. 105, 491, Nov. 15, 1962.
4. R.A. Padilla, C.M. Marrero and A.M. Bonera, Rev. Cubana Farm., 8, 3 (1973).
5. S.A.M. Ali, I. Mahmood, and Q. Khalid, Prep. Biochem., 8, 113 (1978).
6. T.K. Mitrofanova, E. Yu. Bulicher., S.F. Golubiva, T. Yu. Bodina, O.S. Zizina, R.P. Evstigneeva, Deposited Doc., VINITI, 2035-76, 1976.
7. G. Rouser, G. Kritchevsky, D. Heller and A. Lieber, JAOCS, **40**, 425 (1963).
8. O.S. Privett, M.L. Blank, D.W. Codding and E.C. Nickell, JAOCS, **42**, 381, (1965).
9. A. Stachowiak, H. Specht and D. Onken, Ger. Patent, **34**, 176, Dec. 5, (1964).
10. J. Stanley, *Production and Utilization of Lecithin*, in (ed.) Klare S. Markley. *Soybean and Soybean Products*, (Interscience Publishers, Inc. New York 1951) Vol. II.
11. G.G.H. David and J.A.P. David, Fr. Pat. 1, 294, 930 Jun. 1, (1962).
12. H. Besson & Cie., Fr. Pat. 77451 Mar. 9, (1962).