Investigation of Bovine Blood Plasma Utilizing Advanced Identification and Separation Techniques

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SUMMARY

Spray-dried, freeze-dried, air-dried, frozen and liquid bovine plasma (freshly drawn blood which was centrifuged at 1090, 4360, 7740 and 23700 g, respectively) was subjected to chemical/biochemical and separation analyses.

The separation methods employed were UV/VIS spectrophotometry, reverse phase high pressure liquid chromatography (RP-HPLC) and electrophoresis (SDS PAGE). A multiparameter "SMAC"-Technicon Instrument was used for the chemical/biochemical profiles.

Preliminary data (chemical as well as the separation techniques) showed few differences between the fresh plasma samples prepared at the various centrifugation speeds. The spectrophotometric results showed differences between fresh and frozen vs spray-dried and air-dried plasma samples and vs the freeze-dried plasma samples. Marked differences (absence of unidentified absorbing agents) were noted at 412, 463 and 494 nm and a " band shift" occurred, moving absorbance from 300nm to 297 nm, respectively.

Electrophoretic profiles or densitometric data showed no significant differences between the freshly prepared, frozen and freeze-dried samples. Both air-dried and spray-dried plasma samples proved impossible to resolve satisfactorily due to the incomplete solubilization properties of these samples during the electrophoretic run.

The water/acetonitrile (ACN) as well as the phosphate buffer/ACN chromatograms were almost identical. This indicated no significant differences between the heat and non heat-treated bovine plasma samples. The phosphate buffer/ACN system produced the most acceptable separations/peaks.

The chemical analyses showed distinct differences between heat and non heat-treated plasma samples whilst no significant differences were recorded for the plasma samples prepared at different centrifugation speeds. For example, the freeze-dried plasma samples had less sodium, urea, calcium, gamma glutamine transferase, lactate dehydrogenase and cholesterol than the fresh or spray-dried plasma. Significant increases in glucose levels were also recorded for the freeze-dried samples.

It is concluded that the "wild" or "fishy" flavour of powdered blood plasma is influenced, induced and/or promoted by heat. Unidentified, high molecular protein-linked molecules (globulins ?) were implicated.

INTRODUCTION

Food protein sources for human consumption are annually becoming more limited on a world-wide basis. The obvious, but difficult solution is birth control. An alternative approach would be to research and develop alternative protein sources. Much effort has been invested in the following areas: production of single cell proteins; extraction of proteins from seeds, leaves and other plant tissues; edible food waste materials, etc. However, animal blood is very poorly utilised. A limited amount of whole blood, for example in Denmark and Germany has been directly used in human food in the form of black pudding (Alfa Laval Food Engineering, 1985). The remainder is further processed into animal feeds, petfoods and fertilizers. In some instances, the animal blood in the abattoir contributes significantly to the burden and cost of subsequent effluent treatment.

In many countries, whole blood is limited in usefulness for reasons such as religion, colour and flavour but plasma is an almost colourless bland material with qualities that are useful in many types of food products.

The quality of food grade plasma protein would depend upon the practical recovery of animal blood for human consumption. This involves two basic operations, namely aseptic collection and separation of whole blood. The methods by which blood can be collected and further processed have been fully described elsewhere (Alfa Laval Food Engineering, 1985; Gorbatov, 1988). The technology for further processing the red cell fraction of animal blood and its uses is also described elsewhere (Vareltzis and Buck, 1985).

The functional properties of bovine plasma are very similar to those of egg white. Plasma proteins have good solubility, emulsifying capacity, foaming ability, emulsion stability and a very good ability to form gels. Plasma gel firmness is usually used as an indicator of functionality. Gel firmness is dependent upon a number of factors. These include plasma concentration, treatment temperature and duration, pH and sodium chloride. The details regarding blood protein functionality are discussed elsewhere (Autio et al, 1985; Etheridge et al, 1981; Haque and Kinsella, 1988; Hickson et al, 1982; Howell and Lawrie, 1987; Khan et

al, 1979; Lee et al, 1987; Nakamura et al, 1984; Seidman et al, 1979; Shahidi et al, 1984; Terrell et al, 1979; Tybor et al, 1973; Yassuda et al, 1986).

The purpose of this preliminary study was to investigate various separation methods and to identify selected chemical components of the bovine plasma fraction. Little is known about the composition of animal plasma as a process raw material. An attempt was made to identify potential factors that might contribute to the characteristic and possibly objectionable flavour of powdered (dried) plasma (i.e. at concentrations > 2 or more percent dry weight basis).

MATERIAL AND METHODS

The bovine plasma was prepared by pooling freshly collected bovine blood (50L) that was aseptically drawn from several experimental animals. Prior to pooling, the blood was treated with SABAX Transfuso-Vac anticoagulant (SABAX Inc., Deerfield, Illinois USA). Five hundred cm^3 of blood was mixed with 70 cm^3 of anticoagulant (sodium citrate dihydrate (1.84g); citric acid monohydrate (228.9 mg) and phosphate dihydrate (175.7 mg) - all in 70 cm^3 sterile, pyrogen-free water). The pooled blood was separated into plasma and red cell fractions at 4°C by means of centrifugation (i.e. at 1090g, 4360g, 7740g and 23700g, respectively). One litre of the plasma fraction was frozen at -18°C for 2 months, one was cooled at 4°C and the remaining plasma was sub-divided into two fractions. One fraction was dried under vacuum (-70 torr) for 10 days and the other freeze-dried (plate temperature of 20°C and vacuum -70 torr). Prior to analysing the above samples, freshly drawn plasma (prepared by the same methods as above but centrifuged at 4360 g) was included as the liquid control. Fresh plasma (2 L) was also freeze-dried at a plate temperature of < 0°C and a vacuum of -70 torr. Commercial spray-dried blood plasma supplied by the American Meat Protein Corporation (2515 Elwood Drive, Suite 106 Ames, Iowa 50010 USA) was used as the dry/powder control. The moisture content of all the dried samples was < 6%. The vacuum- and freeze-dried samples prepared at 4360 g were used in the analytical tests.

The following standard chemical and/or protein/enzyme tests were conducted by means of a mulitiparameter "SMAC" (Technicon Instruments II, Tarrtown, New York) on fresh bovine plasma separated at different g-values, and 6% (m/v) spray-dried and freeze-dried (centrifuged at 4360 g) test samples. The analytical methods used included the following:- Albumin-Dye; binding-bromocresol green with succinate buffer CV 2%; Bicarbonate: Colorimetric method using phenolphtalene indicator CV 5%; Bilirubin: Suphanilic acid with caffeine-bezoate accelerator CV 5%; Calcium: Colorimetric method using cresolphtalein complexone CV 1%; Chloride: Colorimetric method using mercuric thiocyanate/ferric nitrate CV 1%; Cholesterol: Cholesterol oxidase; Creatine: Jaffe end point reaction; Glucose: Hexokinase; Phosphate: Phoshomolybdate reduction with stannous chloride-hydrazine CV 2%; Potassium: Ion selective electrode CV 2%; Protein: End point biuret reaction CV 2%; Sodium: Ion selective electrode CV CV 1%; Urea: Diacetyl monoxime method CV 2%; Uric acid: Colorimetric uricase-peroxidase CV > 2%; Alanine transaminase (GPT): Rate reaction at 334/340/356 nm, L alanine concentration > 225 mM in tris buffer; Alkaline phosphatase (ALP): p-nitrophenyl phosphate substrate-amino methyl propanol buffer; Aspartate transaminase (GOT): Rate reaction at 334/340/366 nm, aspartate concentration > 100 nM, tris buffer; Gamma glutamyl transferase (GGT): gamma-glutamyl-4-nitro analide substrate; Lactate dehydrogenase (LDH): Lactate pyruvate rate reaction 334/340/366 nm. The intrabatch % CV's for the enzymes were approximately 5-6% whilst the interbatch CV's for the enzymes were approximately 6-7%. Commercial quality control sera were included in each run. The results were accepted only if the quality of the controls were within accepted limits.

All the chemicals and reagents used for the electrophoretic, spectrophotometric and HPLC analyses were of analytical reagent grade or better except the glycerol which was of reagent grade. Water was prepared by a Millipore Milli-Q™ water system.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried on a Bio-Rad Mini-Protean™ vertical electrophoresis unit according to the manufacturers instructions. The total gel size was 7 x 10 x 0.1 cm. A continuous buffer system (Tris, Glycine and SDS) was used with separating gradient gel concentration of 4-20% acrylamide (Bio-Rad catalogue number 161-0903). Two gels were subjected to electrophoresis for 2 h at a constant voltage of 200V. The gels were stained with Coomassie Brilliant Blue R250, destained and scanned with a DU-70 Spectrophotometer. Sample load was varied to optimise resolution.

Spectrophotometer

Known concentrations of plasma protein were scanned spectrophotometrically between 190 nm and 590 nm using a DU-70 Spectrophotometer in scan mode in accordance with manufacturers instructions. The nitrogen content (mgN/100 cm^3) of the samples was:

Nitrogen (mgN/100ml)

Two month-old samples:-

"Fresh"	*1022.5*	*(unfrozen)*
Frozen	*1022.4*	
Air-Dried	*93.9*	*(+/- 1% solution)*
Spray-Dried	*103.6*	*(+/- 1% solution)*
Freeze-Dried I	*115.3*	*(+/- 1% solution)*

Fresh samples:-

Fresh	*1057.5*	*(unfrozen)*
Freeze-Dried II	*189.7*	*(+/- 1% solution)*

Freeze-Dried I dried at 20°C
Freeze-Dried II dried at < 0°C

HPLC

The following equipment was used:
712 WISP (Waters Intelligent Sample Processor)
501 HPLC Pump
510 HPLC Pump
SIM Box (System Interface Module)
490 Programmable Multiwavelength Detector
Digital LA50 Printer
Digital Professional 350 Microcomputer

Columns:

Guard - Supelcosil LC308, C8, 5, 300 Å pore size, 2cm x 4.6mm
Separation - Supelcosil :LC308, C8, 5 , 300 A pore size, 25cm x 4.6mm

Solvents:

Solvent A - 100% Water with 0.1% TFA
Solvent B - 100% ACN with 0.1% TFA

Solvent A - 100% Phosphate Buffer
Solvent B - 100% ACN with 0.1% TFA
(Solvent B must not exceed 70% of total eluent to avoid phosphate precipitation)

Phosphate Buffers:

Sodium Dihydrogen Phosphate 20mM L^{-1} pH 4.75
Disodium Hydrogen Phosphate 20mM L^{-1} pH 9.45

Programs:-

Water/ACN system (Refer to Fig.1)

Time (min)	Flow (cm^3/min)	% A	% B	Curve
0	0.5	100	0	*
5	1	100	0	6
20	2	55	45	3
25	2	40	60	3
30	1	100	0	8
35	0.5	100	0	6

Experimental conditions:

Sample load: 10 µL

Phosphate Buffer/ACN system (Refer to Fig.2)

Time (min)	Flow (cm^3/min)	% A	% B	Curve
0	1	70	30	*
30	1	35	65	8
40	1	70	30	6
45	1	70	30	6

Experimental conditions:

Similar to the water/ACN system. Running time: 45 min

RESULTS AND DISCUSSION

Protein separations by means of the reverse phase HPLC column and the selection of a suitable carrier phase presented several operational problems. Initially, a water/acetontrile gradient system was used. It became clear that inadequate separation was occurring, particularly from 15 minutes onwards. Tailing was also evident. This was theorised as being due to the ionising properties of the proteins in the plasma samples. To overcome this, a phosphate buffer system was tried. This system also proved to be more difficult than anticipated regarding the elimination of the tailing problem. The tailing can be seen at approximately 30 minutes in Figure 1. Varying the pH did little to remove the problem. Above pH 7 poor separations occurred which improved slightly as the pH decreased. With this type of buffer system the best separation that could be obtained (Fig 2.) occurred at pH 4.75 ie. 100% sodium dihydrogen phosphate 20 mM L^{-1} pH 4.75 as solvent A. Attempts to decrease the pH even further may improve the separation. Currently, different methodologies are being tested/pursued to find a suitable buffered system within the constraints of silica column chemistry.

From Figures 3 and 4 marked differences (absence of specific absorbing agents) were noted at 412, 463 & 494nm and a "band shift" occurred between 300nm to 297nm between the fresh, frozen and two month-old "fresh" plasma against the spray-dried, air-dried plasma and the freeze-dried plasma. It was further noted that the freeze-dried plasma gave a UV/Vis spectrum subtly different to the spray-dried and air-dried plasma samples in that the slope of the curve leading to the major peak at 297nm was sharper. No differences were noted between the plasma samples with different g values.

The electrophoretic profile or densitometric results illustrated in Fig. 5 (using a 4-20% gradient polyacrylamide gel in the presence of sodium dodecyl sulphate) indicated no significant differences between the freshly prepared and two month-old "fresh sample", frozen or freeze-dried samples. Both the air-dried and spray-dried samples proved impossible to resolve satisfactorily. The tracks containing these samples illustrated extreme streaking that could not be eliminated with centrifugation (9200 g for 30 min) and/or filtration (0.45 μ diameter membrane/sieve). Such streaking is indicative of either particulate matter (unlikely in view of the above) or incomplete solubilisation/precipitation during the electrophoretic run. The latter is thought the more likely which would indicate that some molecular change(s) has/have occurred to the plasma proteins.

The chemical/biochemical results showed in Table 1 confirmed that (1) the bovine plasma fractions prepared by the different centrifugation speeds of whole blood were very similar

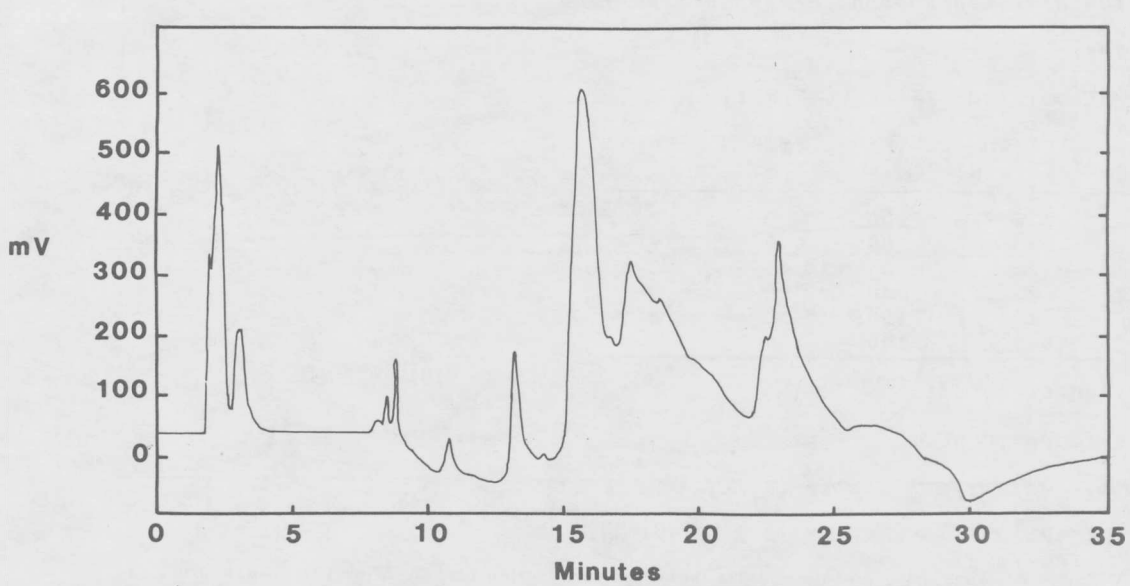

Figure 1. HPLC chromatogram of spray-dried bovine plasma (water / ACN system)

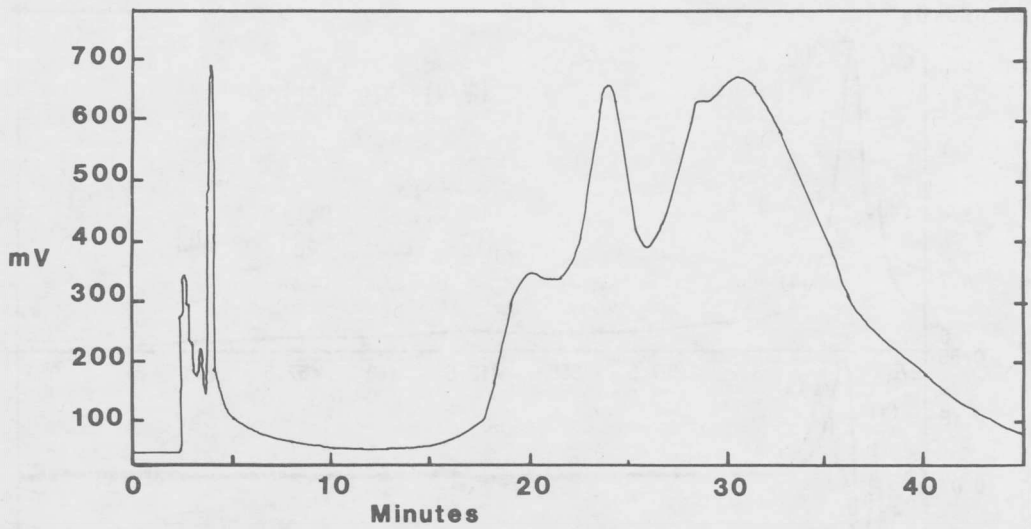

Figure 2. HPLC chromatogram of spray-dried bovine plasma (phosphate buffer / ACN system)

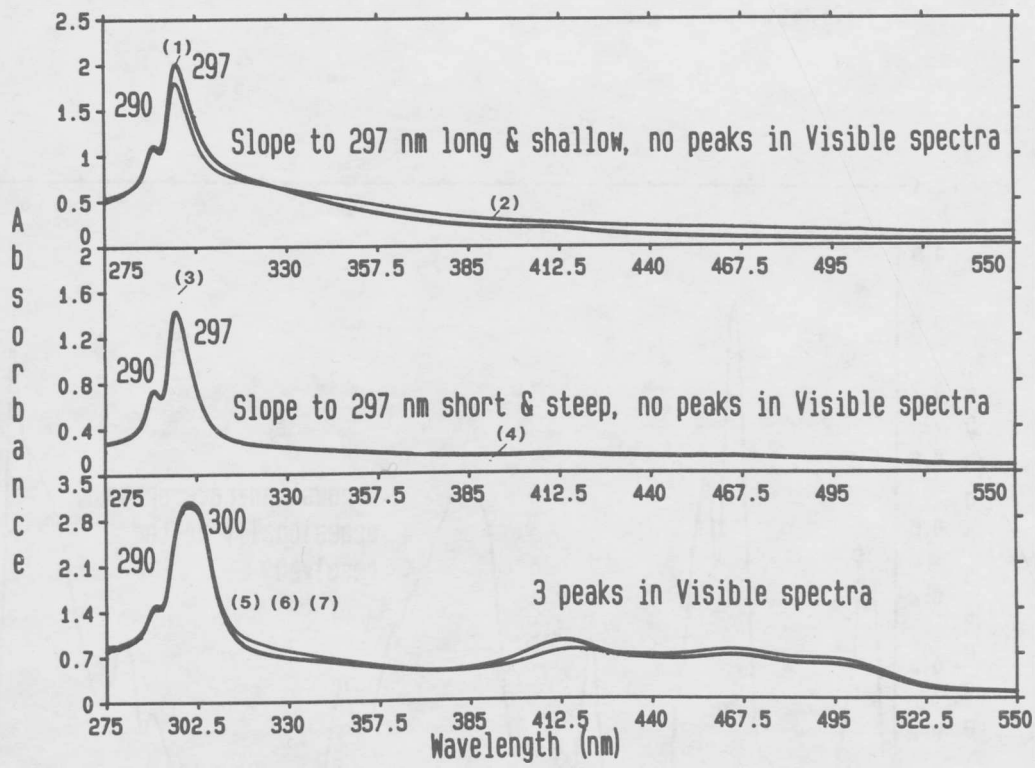

Figure 3. UV / visable spectra (scan) of spray-dried (1), air-dried (2), freeze-dried (at 20°C plate temperature) (3), freeze-dried (at 0°C plate temperature) (4), fresh (5), "fresh" (stored at 4°C for 2 months) (6) and frozen (for 2 months at -18°C) (7) bovine plasma

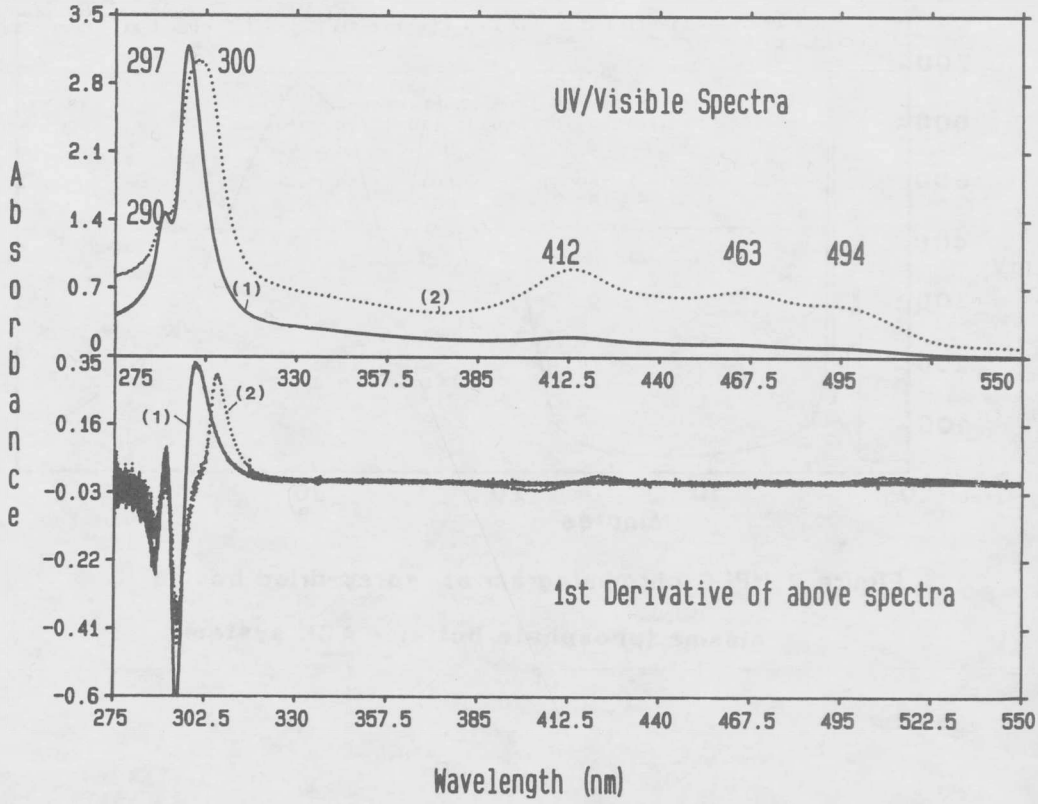

Figure 4. UV / visible and 1st derivative spectra (scan) of spray-dried (1) and fresh (2) bovine plasma

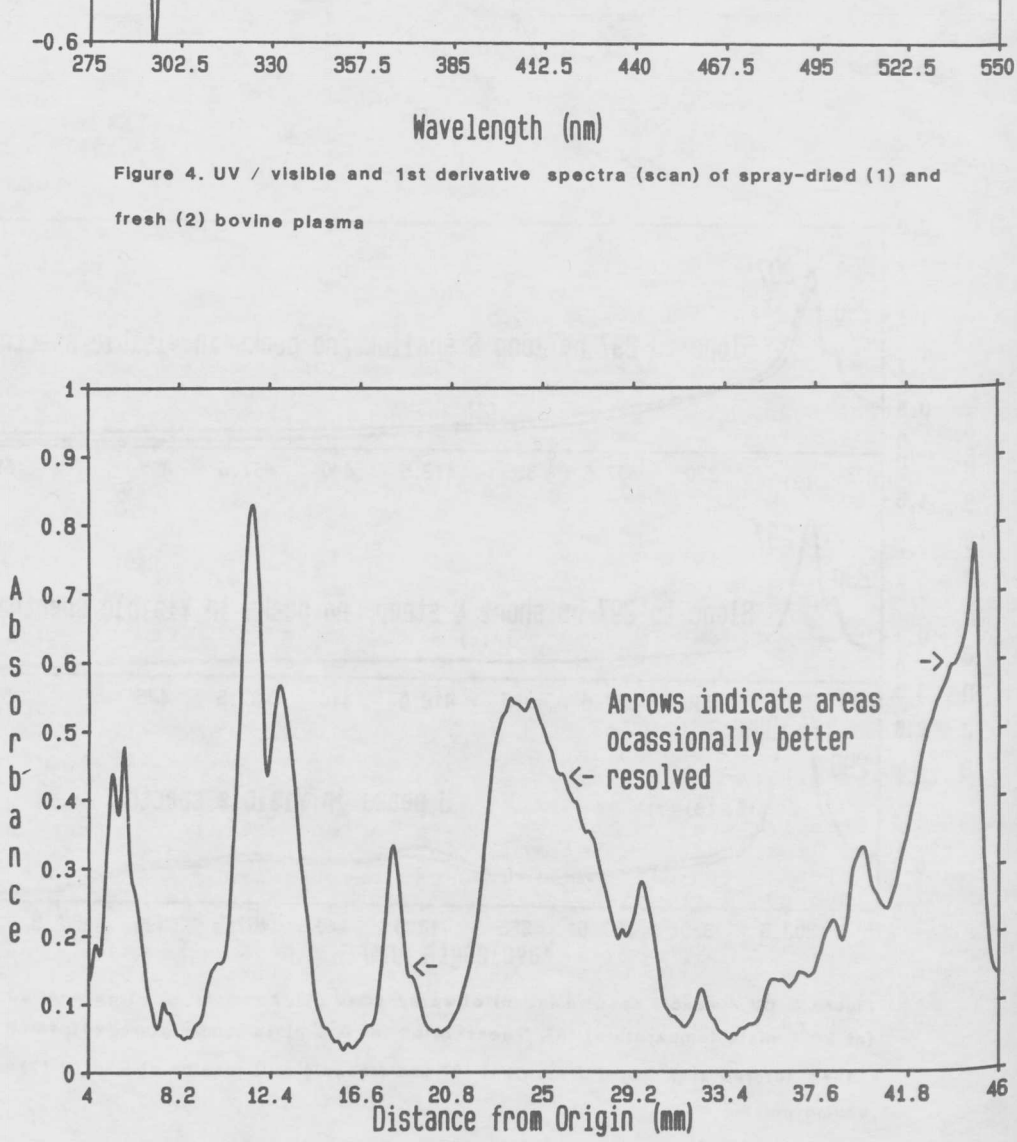

Figure 5. Densitometric spectrum / scan of fresh, frozen and freeze-dried bovine plasma seperated by using SDS-PAGE (4 - 20% gradient, 200V, 2h)

Table 1. Selected chemical/biochemical data on bovine blood plasma

Component	Plasma prepared from whole blood at various centrifugation speeds (rpm) / g values				Powdered plasma	
	(3000) 1090	(6000) 4360	(8000) 7740	(14000) 23700	Spray-dried (6% m/v)	Freeze-dried (6% m/v)
Sodium (mmol/L	139	140	140	140	140	111
Potassium (mmol/L)	4.2	4.3	4.4	4.4	4.4	3.7
Chloride (mmol/L)	101	99	99	100	50	61
Bicarbonate (mmol/L)	23	22	23	22	2.0	2.0
Urea (mmol/L)	7.6	7.2	7.2	7.1	2.7	2.3
Creatine (mmol/L)	74	70	67	76	75	81
Uric acid (mmol/L)	1.04	0.92	0.89	0.88	0.47	0.57
Calcium (mmol/L)	2.17	2.17	2.15	2.19	1.64	1.30
Phosphate (mmol/L)	1.80	1.80	2.06	1.95	1.33	2.47
Protein (g/L)	86	89	87	89	45	42
Albumin (g/L)	--*	--	--	--	24	18
Total Bilirubin (umol/L)	0	0	0	0	0	0
Conjugated Bilirubin (fmol/L)	--	--	--	--	--	
ALP (iu/L)	38	38	38	38	33	44
GGT (iu/L)	32	32	32	33	16	10
GOT (iu/L)	88	87	83	88	14	0
GPT (iu/L)	24	41	22	32	10	15
LDH (iu/L)	788	844	828	865	152	134
Cholesterol (mmol/L)	4.40	4.64	4.58	4.65	2.40	1.20
Glucose (mmol/L)	2.9	1.6	1.4	1.7	1.6	4.4

*--, Not tested

and (2) the fresh plasma fractions differed from the spray-dried and freeze-dried plasma samples. This was particularly obvious when the concentrations of the following components were compared: sodium, chloride, bicarbonate, urea, GGT, GOT, LDH, GPT, cholesterol and glucose.

The UV/VIS spectrophotometric and electrophoretic results and the selected chemical/biochemical data in this study showed solubility, chemical and enzymatic as well as molecular differences between freshly drawn, freeze-dried, frozen and two month-old "fresh" bovine plasma vs spray-dried and air-dried bovine plasma. Whilst the HPLC results showed no differences between heat- and non heat-treated plasma samples, the successful separations by the Supelcosil LC308 reverse columns (refer to Figures 1 and 2), however, indicated no change in the plasma protein hydrophobic properties (it is theorised that this column separates proteins based on their inherent (or non denatured) hydrophobic characteristics. This is a significant observation because the hydrophobic properties of "denatured" proteins are known to affect flavour. Most intact proteins have little flavour but they could influence flavour by binding flavour compounds (Bigelow, 1967). The HPLC results in this study suggested that the "wild" flavour of plasma proteins was probably not caused by protein hydrophobic interactions.

The results in this study confirmed that heat (used/generated during the processing/preservation of liquid plasma) influenced (directly or indirectly), induced and/or promoted the development of the undesired notes in powdered plasma. In support of this view various meat and cake products were prepared with and without plasma powder. Meat (in patty and local fresh sausage formulations) and egg albumin (a variety of cake formulations) was partially substituted by fresh, freeze-dried and frozen bovine plasma and compared to products formulated with and without spray-dried plasma. The concentration of the dried plasma in the formulations ranged from 1 to 5% (m/v) and 5 to 30% (m/v) with "liquid" plasma, respectively (unpublished data). The substitute products (i.e. with plasma at concentrations far greater than what is acceptable by consumers sensitive to the presence of plasma in meat and other food products) were favourably (i.e. > 98%) accepted by the panelists. These results were further supported by presenting the same panelists with similar food products (as indicated above) prepared with air-dried plasma, previously concentrated and dewatered with ultrafiltration and reverse osmosis membranes, respectively. The acceptance by the consumer panelists was equally favourable (i.e > 97% overall acceptance). The air-dried plasma used in the latter experiments were air-dried at 35°C for 3 days instead of 10 days. In addition, the plasma produced under these mild heat conditions was more bland and with texture and flavour qualities similar to the freeze-dried bovine plasma (unpublished data). Unfortunately, the latter was researched months before the current study and was, therefore, not subjected to the same analytical tests. The

quality of the dried plasma powder was probably enhanced by reducing the ash content (i.e by means of the membrane systems).

The presence of glucose (Table 1) in the different treated plasma samples together with the plasma proteins justifies further research in an attempt to examine the significance of the Maillard reaction (non-enzymatic browning) as a means of contributing undesired plasma flavours. This reaction occurs when reducing sugars are present with free amino groups (e.g. lysine). Albumins and globulins could be very sensitive to this reaction (especially in the presence of heat). In practice, a product can be discoloured as well as develop acceptable or unacceptable flavours (Fox and Condon, 1981). This area will be addressed in future research programs. The authors intend treating fresh plasma proteins with glucose oxidase prior to spray-drying or ball-drying (a mild drying technique (< 60°C) described by Nilsson, 1971), after which the dried product will be chemically, physically and sensorically evaluated. According to the study of Morales et al (1976) on the solubility changes of native bovine serum albumin (BSA) and BSA complexed with D-glucose (i.e. by the Maillard reaction), no denaturation was observed below 70°C and a maximum of 70% was reached at 100°C. These reseach workers claimed that the glucose increased the stability of the plasma proteins by protecting them against changes in internal configuration. Furthermore, no visible brown pigmentation was observed after heating BSA and glucose at 55°C for 18 h. These observations suggest that the globulin fraction might be involved with undesired flavour notes. It is a well documented fact that plasma consists of three main groups of proteins: fibrinogen (0.2-0.4 g/ 100 cm^3), globulin (1.9-2.8 g/100 cm^3) and albumin (4.4-5.3 g/100 cm^3). The albumins are water soluble whilst the globulins are salt-soluble (0.3M NaCl). The alpha and beta globulins contain fractions of lipoproteins (Price and Schweigert, 1971). The involvement of fibrinogen proteins (i.e. with off-flavour development) is unlikely, if they are sucessfully removed during centrifugation. The importance of researching the various plasma fractions further is accentuated by the information reviewed by Lawrie (1982). In his review on the isolation and utilization of food proteins, Lawrie (1982) summarised the findings of Howell regarding the gelling, foaming and emulsifying properties of whole blood plasma and the three plasma fractions prepared by means of DEAE-cellulose chromatography. When the different fractions were added to a cake-type model system, at different times and heating temperatures, the "fishy" odour which tends to develop during storage of whole blood plasma appeared to be absent from the three separate plasma fractions. The crux of this study was that intensive interaction between the proteins present in the other ingredients and those of the various plasma fractions, took place. These interactions differed in nature and degree, as reflected by the various organoleptic parameters of this study. It could be speculated that some of these interactions could cause plasma proteins to undergo conformational changes and/or partial proteolysis. For example, partial proteolysis of casein, soy and gluten is known to yield bitter peptides. The bitternes of these peptides is related to their net hydrophobicity or apolar character (Lawrie, 1982).

Lipoproteins consist of triglycerides, free and esterified cholesterol and phospholipids. The presence of cholesterol is indicated in Table 1 (refer to the review of Gorbotov (1988) and Donnelly et al (1978) for additional information on the chemical composition of whole blood and blood plasma of domestic animals) and at different concentrations in the fresh, spray-dried and freeze-dried plasma samples. These polar lipids are known to be sensitive to oxidation and impart a "warmed-over flavour" (WOF) to food products within 48 h (Pearson, Love and Shorland, 1977). The exposure of plasma proteins to heat might induce a similar WOF-type of reaction(s) that may lead to undesired flavours. The inclusion of suitable chelating agents and anti-oxidants to fresh plasma prior to air-drying and spray-drying, is currently being investigated as a potential means of eliminating the wild flavour of dried plasma proteins. In addition, trace amounts of haemoprotein (due to inadequate centrifugation of red cell fraction and its subsequent haemolysis) and lipids in the plasma fraction could lead to undesired flavours. Intimate contact between the haem proteins and the lipids could probably contribute to the development of both oxidative rancidity and discolouration. Hence, the importance of centrifuging whole blood under the correct conditions. Great success in our laboratory has been achieved with the separation of the red cell fraction by means of inorganic membranes.

CONCLUSIONS

The UV/VIS spectrophotometric and electrophoretic results and the selected chemical/biochemical data in this study showed solubility, chemical and enzymatic as well as molecular differences between freshly drawn, freeze-dried, frozen and two month-old "fresh" bovine plasma vs spray-dried and air-dried bovine plasma. In addition, the data in this preliminary investigation confirmed that heat (used/generated during the processing/preservation of liquid plasma) influenced (directly or indirectly), induced and/or promoted the development of the undesired flavour notes in powdered plasma.

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