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"Bioactive Choices, Business Models and Breakthrough Technologies for the Sustainable Manufacturing of High-Value Added Bioactive Products from Red Meat Sources: Part I".

INTRODUCTION: Human evolution has been totally dependent on the ability of individuals to achieve a satisfactory state of nutrition and well being. Central to these requirements has been the availability of biological substances, many of which today are called in lay terms "bioactive compounds". These substances are derived from microbial, plant or animal sources, and thus can be considered to represent the commercially valuable product outcomes of more renewable resource manufacturing industries than alternative types of synthetic products generated from the petrochemical industries.

In scientific and process engineering contexts, industry has understood for more than two hundred years the economic potential and commercial value of individual "bioactive compounds" that can be obtained from the first two of these sources, although clearly the nutritional and health benefits of substances derived from microbial or plant sources have been imbedded in human folk-law and experience for several millennia. Today, major multi-billion dollar businesses have been created around these opportunities through the implementation of large scale fermentation technologies, multistage extraction and purification methods, robust product quality control procedures and extensive scientific evaluation of the nutritional, biological and epidemiological advantages of these substances and materials.

As a consequence, many "bioactive compounds" derived from microbial and plant sources have found or are now finding their way as mixtures into functional or fortified food products as additives and ingredients, or as popular single use nutraceutical products. In practice, these commercial developments have had as their basis a long scientific and engineering heritage, which bridges multiple areas of human knowledge, technical experience and the operational practices of many industrial organisations and market sectors. For these reasons it is not surprising that bioactive compounds, such as vitamins, e.g. vitamin C or folate; essential minerals, e.g. calcium, iron or zinc salts; carotenoids, e.g. β-carotene or lycopene; or other substances, e.g. L-carnitine, polyunsaturated fatty acids or coenzyme Q, all represent in their own right major commercial opportunities because of their important biological properties in delivering good health.

In each of the above cases, the "breakthrough" leading to their development and commercialisation has hinged on the discovery of advanced processing technologies and the associated knowledge on how to make, deliver and assess the biological efficacy, safety and application scope of these products. It is also not surprising that with the commercial maturity of these products, new ways to carry out business have also emerged, involving different companies networked to operate with different expertise within the same or different business supply chains. Significant paradigm shifts in product opportunities, their manufacturing processes and marketing strategies have thus emerged in recent years with these microbial and plant derived bioactive compounds.

These developments have also acted as the business catalyst for the strategic establishment of a variety of commercial alliances of different magnitudes, critical mass and impact. Extensive reports generated by these alliances have detailed the emerging trends where supply chain integration related to products derived from microbial and plant sources have been achieved. The European Responsible Nutrition Alliance (ERNA)[1] -- a consortium of very large companies encompassing the chemical, food product supplier and processing industries, including *inter alia* BASF, Lonza, DSM Nutritional Products, Novartis Pharma and other large corporations interacting as a single alliance -- is representative of these partnerships. Importantly, such alliances operate as transnational entities with activities that extend across an entire field, e.g. ERNA's activities deal with the manufacture and marketing of food supplements to enhance a healthy diet through a common approach that reflects the interests of both the consumers and the industry with the ability to act as a "one stop" entity to scientifically and technically inform regulatory/legislative bodies – rather than as a entity with a single product or single market segment as their raison d'etre. Such coordinated approaches provide important components of the road map and capabilities needed for the future development of other innovative products and the commercial opportunities that they represent. Moreover, the extensive base of expertise, technical know-how and critical mass that is captured through these supply chain alliances has enabled other areas of business opportunity to prosper. For example, in an analogous manner different areas of the chemical and industrial biotechnology sector involving manufacturers, suppliers and marketers which historically have been distally or competitively placed in terms of their location in a supply chain have come together to deliver commercial outcomes with other classes of high value products, e.g. the Dupont-BP Advanced Biofuels Partnership[2] or the Dow-Saudi Aramo Sadara Initiative[3] linking 25 other business units as a world scale chemicals alliance with forecasted annual revenue of $20B.

In comparison, the level of commercial activity and supply chain network co-ordination associated with the co-generation of high value added bioactive products from red meat animal sources has generally not reached, with some notable exceptions, e.g. several "bioactive" products generated from waste streams within the dairy industry, a similar stage of maturity in terms of

(a) the levels of innovation that have been implemented to enable the production of high value added bioactive products to be achieved with very efficient processes at a scale, flexibility, cost competitiveness and robustness that matches commercial realities.

(b) access to technological platforms and engineering solutions to permit multiple products to be generated from the same feedstock in volumes compatible with the requirements of servicing significant market sizes.

(c) the integration of the capabilities of different industry partners into alliances that bridge the expertise, human resource capacity and technical skills of the participating organisations, often located at different places within different (or

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1 Report of the European Responsible Nutrition Alliance (ERNA) Facts about vitamins, minerals and other food components with health effects. (2011) ISBN 9789081 760225 pp 1 -101.

2 Semans, T. and de Fontaine, A. *Pew Centre Report* (2009) http://www.c2es.org/docUploads/Climate-Tech-JV-Strategy_DuPont-BP_sept-2009.pdf

3 http://sadara.com/en/

even the same) supply chains, in order to synergistically deliver multiple, commercially viable technological and business solutions and products that are required to sustain a significant industrial sector.

(d) the level of understanding of the linkage between available innovative production technologies needed to manufacture bioactive compounds, their use initially in addressing the consumer's need for safe and effective additives and ingredients (often but not exclusively as nutritional enhancers) in food products that improve human well being, and subsequently for use in underpinning the evolution of more sophisticated bioactive products of considerably greater commercial value and market interest.

The question is why have similar alliances for the consortium-based production and global marketing of red meat derived bioactive products not emerged to the same extent in terms of operational scale and critical mass of skills, particularly in a country like Australia where the resource feed stocks are available in relatively high abundance and with high (bio)safety quality. In order for this question to be placed into context *vis-a-vis* recent developments in the identification, prioritisation, processing, marketing and commercial delivery of bioactive products derived from other types of feed stocks, attention has to be focused on the molecular nature, functional attributes and intended uses of the high-value added products that potential can be obtained from red meat sources.

Moreover, consideration has to be given to the type of business models that currently exist or could conceivably be developed in the near future. With this understanding, a number of 'low hanging' opportunities of relatively short time horizons can be prioritised, and the business case for dealing with longer term opportunities better evaluated. In addition, the scope of breakthrough technologies that could form the basis of a sustainable "bioactives" industry in Australia needs also to be considered, taking into account the nature of supply chain trends in other areas and the associated technologies and processes employed.

Recognising the need to achieve critical mass in terms of the technical skills and infrastructure that are required, MLA has surveyed the opportunities for bioactive products, resulting in the generation of a *MLA Bioactives Compendium*[4] prepared to enable red meat processors and value adders with a ready reference about the various bioactive products that may be derived from red meat sources. Following on from this joint initiative with the Australian Meat Processor Corporation (AMPC), MLA has selected an initial group of five bioactive candidates with large global markets as potential opportunities for the Australian industry.

In this article (Part I), emphasis has been placed on the inter-relationship between product choice, feedstock selection, process design, and the manufacturing and business paradigms that are increasingly dominating other sectors of the 'bioactives' market, whilst the subsequent three articles (Part II-Part IV) will examine several scenarios related to the deployment of existing and emerging technologies, e.g. the use of disposable, low cost single use technologies[5,6] or the down-stream membrane

[4] http://www.redmeatinnovation.com.au/project-reports/report-categories/co-products/bioactive-opportunities-for-the-australian-red-meat-industry, (2011) ISBN 9781741915457, 1-27.

[5] Langer E.S. *Genetic Engineering and Biotechnology News*, (2011) **31**, 18.

adsorbent systems being popularised in the field of industrial biotechnology. In addition, the inherent advantages of using the same platform technologies to process the same feed stocks, suitably collected, to allow multiple products to be obtained with the same level of infrastructural investment and staff skills will be explored, drawing on the analogous cases that exist in the field of human biopharmaceutical manufacture and the production of low molecular weight bioactives from microbial and plant sources.

Subsequently, in Part II-Part IV relevant information will be summarised on the development of new procedures for the extraction of red meat bioactives, where the use of benign solvents, super- or sub-critical CO_2 or water could play a significant role, and recent advances in the processing and purification of bioactives using either new types of adsorbents configured in micro-particulate and membrane formats or two-phase extraction procedures. The underlying assumption that has been made in preparing this and the subsequent articles is that the teaching and experience gained over many years in technically similar, but often not product-associated, industries, such as the biopharmaceutical, chemical processing and functional food processing industries, are highly relevant to the emergence of an Australian red meat bioactive industry, but that these developments are often not fully understood or appreciated by current management, stakeholders and staff working within the Australian farming, abattoir or meat processing communities.

PRODUCT SELECTION, PROCESS DESIGN AND MANUFACTURING PARADIGMS:

Over the past decade, health conscious consumers in many countries have increasingly sought access to functional foods and other products that contain one or more bioactive substance with beneficial attributes. Such chemical or biological bioactive substances are often called nutraceuticals, and have frequently been associated with vegetable or milk-related products. However, increasingly, the potential for using red meat-based bioactive substances with significant physiological or other functional properties has been recognised both by industry and governmental agencies. For example, in recent years, countries with advanced economies, such as Japan, United States, Europe, etc, have all sponsored and undertaken major programmes of research, evaluation and commercial development of many of the low molecular weight chemical compounds found in red meat.

As a consequence of these developments, a diverse range of consumer products, containing for example conjugated linoleinic acids (CLAs) with anti-carcinogenic and anti-oxidative properties, anti-oxidative histidyl dipeptides such as carnosine (β-alanyl-L-histidine) or anserine (N-β-alanyl-1-methyl-L-histidine), or the anti-cholesterogenic and anti-apototic L-carnitine (β-hydroxy-γ-trimethylamino butyric acid) as representative exemplars, have been approved for human consumption. Many of these substances are now widely available as nutraceutical additives and supplements and marketed in the United States and elsewhere as, for example, drink products for "elite athlete use" and associated with multi-million dollar per annum

[6] Sandle, T. and Saghee, M.J. *J. Commercial Biotech.*, (2011) **17**, 4.

commercial activities and businesses. In the majority of cases, the claimed beneficial effects of these bioactive products have been supported by detailed, scientific peer-reviewed biological, physiological and chemical data. Included amongst these claimed health benefits have been bioactives with anti-inflammatory, immuno-modulatory and anti-microbial activity with their use targeted to prevention rather than to treatment of a disorder[7]. The roles of some of these low molecular compounds in human health, and the manner of their commercial exploitation, have been the subject of a variety of recent reviews and detailed reports[1,8,9,10,11].

In comparison, much less effort has been expended by industry and the relevant governmental funding agencies to capture a similar level of extensive commercial opportunity of the mid to high molecular weight substances, typically associated with peptide, proteins, polysaccharides and their derivatives, present in feed stocks derived by the red meat industry. Typically, the products that have been generated are often been considered as one-off structurally well characterised bioactive targets, such as bovine albumin, chondroitin sulphate or a specific serine proteinase, such as trypsin, with the manufacturing benefits that could have been gained from operating within a coordinated and fully integrated supply chain model uncoupled or partial uncoupled leading to higher unit product costs.

Therein lays the challenge and the opportunity. Over the past years, it is apparent to numerous workers in the field that a shift in mind-set has prevailed from the level of senior management down to junior staff in how manufacturing approaches and supply chain opportunities have been utilised within sectors of the bioactives industry associated with the production of high purity low molecular weight bioactives obtained from plants, such as the phytosterols, polyphenols and other anti-oxidant compounds. Similar trends with changes to the traditional manufacturing practices and staff culture have also occurred within the biopharmaceutical and industrial biotechnology industries. Can these trends and experiences be replicated for bioactives obtained from red meat sources? If they can, it is highly likely that a similar shift in business practices will occur and the essential driver for the emergence of an Australian industry based on red meat bioactives, taking advantage of the availability of an abundant supply of appropriate, high quality and safe feed stocks and innovative process technologies.

What are the overarching drivers for these trends and their associated technological solutions? Simply stated, it is the transition from the old way of capturing and delivering business opportunities, whereby product development and manufacturing were traditionally dominated by the balance between cost and performance. In this traditional scenario, business sustainability, in its broadest sense, was captured largely through minimising the cost of both materials and equipment infrastructure. Incremental rather than step changes in technology became the norm with competitive advantage rarely measured in terms of product innovation and enhanced

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7 S. Ghosh and R. J. Playford *Clinical Science*, (2003).**104**, 547–556.

8 Arihara, K. and Ohata, M. in F. Toldr´a (ed.), *Meat Biotechnology*, Springer Science+Business Media, LLC (2008), 231-249.

9 Dugan, M.E.R; Aldai, N; Aalhus, J.L; Rolland, D.C. and Kramer, J.K.G. *Can. J. Anim. Sci.* (2011) **91**: 545-556.

10 Milner, J.A. Report to the American Society for Nutritional Sciences - *Molecular Targets for Bioactive Food Components* (2004) 2492S-2498S.

11 Vercruysse, L., Van Camp, J., and Smagghe, G. *J. Agricult. Food Chem.*, (2005). **53**, 8106-8115.

process productivity.

This traditional approach, leads to a number of important sequelae, including the constraints imposed by a linear model of manufacturing, commonly known as the "Cradle-to-Grave Approach"[12] (cf: Figure 1). Derived from the practices of life-cycle analysis and codified as product stewardship components and assessment procedures of ISO 14040-14049 Principles and Guidelines[13], this concept captures the entire life trajectory of an individual material or product from creation up to the point of its destructive disposal. However, it provides little or no guidance on business or product sustainability.

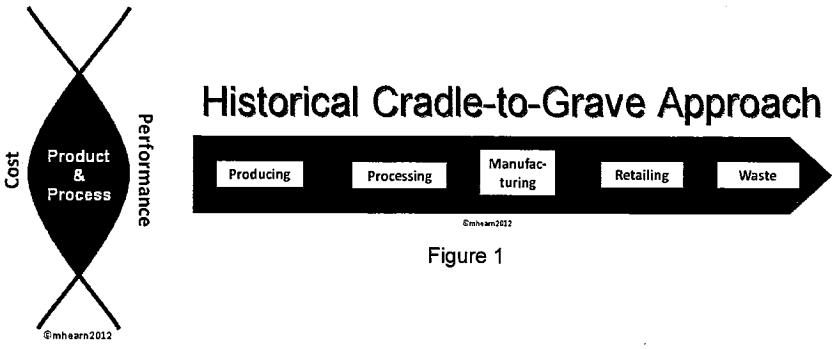

Figure 1

As a consequence of the globalisation of economic activity and the workforce over the past decade, when this traditional approach is deployed any competitive advantage previously accrued from achieving cost-of-goods minimisation will increasingly become constrained with the margins and profitability of primary products generated by such linear manufacturing processes at risk if the full value of co-products is not captured. Despite the fact that world consumption of meat products is estimated to reach 40 kg per capita by 2020[14], a similar trend of constrained margins is likely to occur in the future for the red meat industry where the primary products have historically been meat for direct human consumption. For this reason alone the need to better capture the full value of co- or secondary, products is self evident. Moreover, when the "Cradle-to-Grave Approach" is employed, the methods of delivery of the primary product(s) in terms of the producer/suppliers' capabilities, seasonal availability, climate variability and the manufacturers' procedures have often become uncoupled from those needed to obtain high value-added secondary products at scale in an economically viable supply chain sense.

Thus, when the "Cradle-to-Grave Approach" is applied to red meat products, the generated meat waste, offal and blood are perceived to be of low or no value and, in many cases, have been treated as an intangible cost burden due to the need for disposal or rendering or the need to meet environmental/regulatory compliance requirements and transport/collection issues, rather than routinely being considered as an essential feed stock or raw material for the production of valuable secondary products. When these products are protein bioactives, on a weight basis they are often considerably more valuable than the primary product. Such is the case, for example, of the industrially and diagnostically important enzymes lactate dehydrogenase (LDH) and malate dehydrogenase (MDH), present in bovine heart

12 Dhillon, B.S. *Life cycle costing for engineers*, CRC Press, Boca Raton, USA, (2010) 1-204

13 http://www.iso.org/iso/

14 Delgado C.L., Courbois C.B. and Rosegrant M.W. Global food demand and the contribution of livestock. International Food Policy Research Institute (IFPRI). MSSD Discussion Paper No. 2, p. 1-36; von Braun, J., Food-Security Risks Must Be Comprehensively Addressed. Annual Report 2008–2009, doi: 10.2499/0896299236AR0809E, 1-12.

waste, and purified by a common downstream processing platform technology. Similar considerations and circumstances apply to many other types of bioactives.

As noted earlier, within a number of complementary industry sectors that deal with bioactive products generated from microbial, vegetable/plant and even fish sources, the guiding determinant has increasingly been how best to capture the benefits of an integrated supply chain. Increasingly, this is achieved through operating through a highly organised, well net-worked alliance or consortium structure. In this way, the material and energy flows through the industrial processing system can be optimally employed with minimal losses, thus better achieving more sustainable product development. Business models and the characteristics of their scientific and engineering solutions have, as a consequence, changed to accommodate these benefits. From the various case studies that are now available, adoption of this so called "Open Loop Cradle-to-Cradle Paradigm"[15], schematically shown as Figure 2, has led to significant improvements in the ability to identify product opportunities, to deploy innovative methods of manufacture, and to generate greater productivity coupled with improved profitability margins for products obtained from low cost feed stocks.

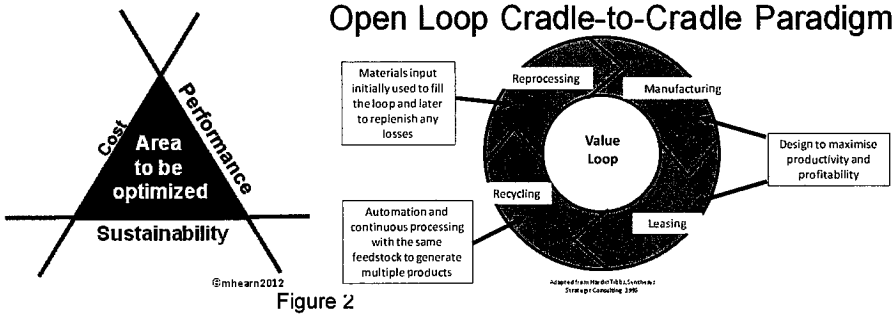

Figure 2

Importantly, in this modality of the "Open Loop Cradle-to-Cradle Paradigm" production optimisation and intensification takes into account the products' total environmental footprint, the energy, water and resource balances, and full system analysis metrics, including CAPEX, OPEX and other key operational parameters which have cost or performance sensitivities effects on the product (and business) sustainability, i.e. goes well beyond traditional life cycle analysis (LCA) methods.

Because of the cost savings, higher efficiencies, better yields, less waste generation and the ability to deploy innovative processing methods, it is not surprising that the industry sector that usually is considered to be at the top of the supply chain pyramid of bioactive compounds -- the pharmaceutical industry -- has placed great emphasis on the adoption of the "Open Loop Cradle-to-Cradle Paradigm" as an essential feature of the manufacture of active pharmaceutical ingredients (APIs). For example, adoption of this approach enables the full spectrum of knowledge from the stage of descriptive understanding, e.g. is a specific compound soluble in a particular solvent?, through to a detailed design understanding based on *de novo* first principles (cf. Figure 3 below) to be built into the process design, and presented as a set of options of optimised process alternatives.

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[15] McDonough, W., and Braungart, M., *Cradle to Cradle: Remaking the Way We Make Things*, North Point Press, New York (2002) 1-208, ISBN: 9780865475878; Lovins, L.H., Rethinking Production, In *Innovations for a Sustainable Economy* (2008) 32-49 http://www.worldwatch.org; Allenby, B., The ontologies of industrial ecology? In *Progress in Industrial Ecology,* Inderscience Publishers, geneva, Switzerland, *(2006) 3;* Frosch, R.A. and Gallopoulos, N.E. "Strategies for Manufacturing". *Scientific American* (1989) **261,** 144–152, doi:10.1038/scientificamerican0989-144.

These approaches enable process systems to be interrogated either in terms of scaling metrics derived from existing engineering flow diagrams or alternatively through the use of "retro-engineered" or "reverse engineered" flow diagrams with process intensification parameters directly incorporated. In this manner, rigorous analysis of the impact of the scientific, engineering and economic parameters on the efficiency of a process intensification method can be carried out and the levels of manufacturing science needed for product realisation and sustainable development more comprehensively determined[16]. Significant financial benefits, amounting to many $10Ms per annum per product have been realised when such procedures have been applied to API manufacture[17]. Other industry sectors further along the chemicals/biologicals supply chain -- such as a red meat bioactives industry -- may not currently need the same level of sophistication as the pharmaceutical industry in terms of the stringency of their GMP manufacturing requirements and regulatory approvals, but nevertheless similar principles and concepts will increasingly become relevant.

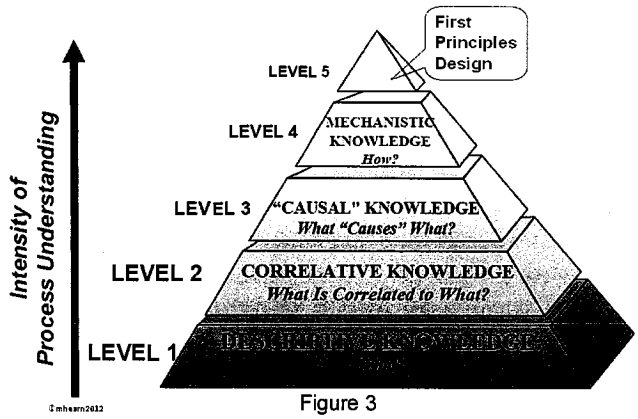

Figure 3

In addition, these integrated technologies also enable feed stock sourcing and processing of a specific bioactive product to be better identified within supply chains that are capable of generating multiple value-added products from the same feedstock. This approach, although not yet fully understood or utilized by many businesses -- the high-end industrial biotechnology and biopharmaceutical industries being the exception -- goes well beyond the concept of sustainable development as introduced by the Brundtland Commission[18] in 1987. Should a similar manufacturing paradigm be deployed for bioactive products from red meat-derived feed-stocks, opportunities implicit to their commercial production and marketing can be evaluated within a practical and readily implemented framework in a manner analogous to that used in other sectors of the bioactives and chemical industries, with the assumptions behind the business case interrogated in detail.

Already a number of organisations based overseas, for example, in Canada, Europe, Ireland, Japan, New Zealand and USA, and supported by national government and strategic business development initiatives[19], have recognised this potential, and have

16 Larson. A., Archer, G., White, M. and York, J. Harvard Business Review, (2006) **6**, 1-4.

17 Cue, B. Vice President, Pharmaceutical Sciences, Pfizer Global R&D, Groton, CT USA; US Pharmaceutical Roundtable Co-Chair, Washington, DC USA. (2012) Personal communication.

18 Report of the World Commission on Environment and Development: Our Common Future; Annex to document A/42/427 United Nations General Assembly (March 20, 1987). http://www.un-documents.net/ocf-02.htm.

19 See for example (a) Functional Food Research and Development Plan, Agriculture and Agri-Food Canada, http://www.agr.gc.ca, http://www.agr.gc.ca/aliments/nff/ffnmarket/ffnmrkt.html, KPMG Report "Canadian Technological Roadmap on Functional Foods and Nutraceuticals.pdf" (2002) pp 1-235; (b) Irish Department of Agriculture, Fisheries and Food National Development Plan 2007-2013. http://www.agriculture.gov.ie/; (c) Japanese Ministry of Health and Welfare FOSHU Initiative, http://www.mhlw.go.jp/foodsafety/fhc; (d) NZ Ministry of Science and Innovation, "Biotechnologies to 2025" Roadmap,

established programmes to develop a number of targetted bioactives from feed stocks which hitherto would have been considered to be low value waste. Within Australia, MLA has also shown leadership through a series of Workshops[20] and other activities with the objective to identify specific bioactives, their production and their route to market. To date, a compendium of bioactives has been generated, with a priority list of five bioactives (bovine serum albumin, bovine immunoglobulins, bovine (pro-)thrombin, bovine haemoglobin and bovine chondroitin sulphate) determined[4,21].

What are the feed stocks from which these valuable bioactives can be obtained? The major feed stock opportunities relate to red meat(muscle), blood (plasma), bone and connective tissues. The question is whether the targets should be of extremely high value, which frequently equate with very low abundance substances – the so called high handing "fruits" which possible could find their way into a medical rather than a nutritional applications, or alternatively should the targets be of intermediate value with a significant scale of use, and readily obtained in relatively high abundance. The five bioactives recently prioritised[4] by MLA from the *Bioactives Compendium* fall into the latter category.

There are, however, other categories of bioactives with even larger scales of use that can be considered as attractive candidates -- nutraceutical ingredients/additives for application in functional or fortified foods. Although it is feasible to contemplate the production of specific, very high value bioactives with medical or veterinary application endpoints in mind, such as cytokines, growth factors, complex polysaccharides or other bioactives with profound physiological properties, often their abundances in tissue of fluid sources are very low – with extraction/purification factors of the order of 25,000-250,000 required if the specific bioactive is to be obtained in high purity and functionality. Moreover, the time lines and costs to achieve regulatory approvals for the medical or veterinary use of such bioactives are generally very significant. In contrast, if the focus was on low hanging "fruits" such as functional food ingredients then the path and cost to market becomes considerably more straightforward, particularly if the bioactive can be readily liberated from latent, high abundance and readily obtained macromolecular precursors.

Thus, the enzymatic hydrolysis of readily available muscle proteins leads to the generation of a variety of bioactive peptides with significant antihypertensive effects as angiotensin I-converting enzyme (ACE) inhibitors[22,23], whilst from other tissue sources, such as the lower value off-cuts and offal from red meat animals following slaughter, bioactive proteins, peptides and other molecules with immuno-modulatory, hypocholesterolaemic, opioid, anti-thrombotic, anti-oxidative and antimicrobial properties can be obtained in yields compatible with their viable large scale production and use as nutraceutical additives in functional foods and other value-

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http://www.msi.govt.nz, http://www.nzbio.org.nz; (e) USDA National Institute of Food and Agriculture (NIFA)-Agriculture and Food Research Initiative (AFRI), http://www.usda.gov/

20 MLA Workshop Reports: 'Realising the potential' 25 November 2005; 'Evaluating our opportunities', 1-2 June 2006; 'Bioactives – Bioprocessing cost estimation', 19-20 April, 2007; 'Seizing the Opportunity', 9-10 October 2009; and 'We can do this', 11-13 October 2011.

21 http://www.redmeatinnovation.com.au/innovation-areas/value-adding/bioactives.

22 Arihara, K. *Meat Science*, (2006) **74**, 219–229.

23 Ryan, J.T., Ross, R.P., Bolton, D., Fitzgerald, G.F. and Stanton, C., *Nutrients* 2011, *3*, 765-791.

added products[24,25,26,27].

Although the molecular structures, such as amino acid sequences, are already known for a number of these molecules, considerable scope still exists to develop advanced processing technologies, with a high level of IP and technical know-how protection. For example, such developments would enable the cost-efficient and scalable recovery and production of biologically active peptides from red meat waste streams, based on for example the use of a cocktail of proteolytic enzymes or by controlled microbial fermentation techniques, to exploit the biocatalytic potential of these systems at a process scale. By taking advantage of the benefits of a consortium or alliance business arrangement, the technical skills base and plant infrastructure already exists in Australia to a significant extent. Moreover, a significant technical and competitive advantage could be captured in a manner similar to that already achieved by other sectors of the bioactives and industrial biotechnology industries, through application of recent advances in 'at-line' process analytical technologies (PATs), coupled with the advantages offered from directed enzyme evolution and selection technologies, to deliver bioactive molecules at a process scale compatible with either direct use or following formulation incorporation into functional foods as high value ingredients/additives once the relevant regulatory approvals for consumer use as food products have been obtained.

Similar processing technologies to those described above are equally relevant to the production of collagen hydrolysates from waste skin/hides[28] as well as human consumption-grade gelatins, and chondroitin sulphate related polysaccharides from similar connective tissue sources[29]. The use of related biocatalytic systems is also relevant to the processing of other feed stock materials, such as the bovine globin pool derived from bovine blood, to generate lower molecular weight fragments that can be utilised to modulate the emulsification properties of protein supplements with functional properties or for special dietary use as 'beverage formulations" for individuals with nutritional deprivation. In parallel to the capabilities developed by the dairy industry to use spray drying technologies to remove the water (often the largest contributor the waste burden, the mass intensification index, and the energy burden during process intensification) can be deployed for facilitate the harvesting and packaging the bulk formulation as solid powder products.

For bovine protein-based bioactives, the application of bio-informatic tools and associated databases, such as UNIPROT[30], BIOPEP[31], NCBI-PROTEIN[32] or EXPASY PROTEIN PORTAL[33], has considerable potential to facilitate product

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24 Korhonen, H., and Pihlanto, A. in Y. H. Hui (Ed.), *Handbook of food products manufacturing – Health, meat, milk, poultry, seafood, and vegetables*. Hoboken, NJ: John Wiley & Sons (2007) 5-37.

25 Gobbetti, M., Minervini, F. and Rizzello, C. G. In Hui Y.H. (Ed.), *Handbook of food products manufacturing – Health, meat, milk, poultry, seafood, and vegetables*. Hoboken, NJ: John Wiley & Sons (2007) 489–517.

26 Hannu Korhone, H, and Pihlanto, A., *Int. Dairy J.* (2006) **16**, 945–960.

27 Nagaoka S. In: Mine Y and Shahidi F. (Eds) *Nutraceutical Proteins and Peptides in Health and Disease.* Boca Raton: Taylor and Francis, 2006: 41-67.

28 Galea, C.A.; Dalrymple, B.P.; Kuypers, R. and Blakeley, R. *Protein Science* (2000), **9**, 1947-1959.

29 Nakano, T., Betti, M. and Pietrasik, Z. *Food, Nutrition and Agriculture* (2010), **2**, 61-74.

30 http://www.uniprot.org/

31 http://www.uwm.edu.pl/biochemia/index.php/pl/biopep/27-list-of-publications-concerning-biopep-database

32 http://www.ncbi.nlm.nih.gov/protein

33 http://expasy.org/links.html

identification and the design of the process selection rules. For example, it is now feasible to perform *in silico* studies with bovine meat proteins as potential precursors of biologically active peptides, as well as to determine whether these peptides can be released selectively by appropriately chosen proteolytic enzymes[34,35]. Illustrative of this potential in product identification and design of the selection rules for process intensification are the data shown in Table 1 below. In this manner, the preferred source(s) of the desired bioactive products can be rapidly identified from this *in silico* assessment, and possible biological attributes of the various target bioactives anticipated by reference to other information held within the data base or alternatively correlated with experimental data acquired with, for example, high throughput screening techniques [now increasingly based on automated robotic liquid handling microfluidic equipment]. Moreover, related tools based on tissue specific proteomics methods similar to those employed[36] to generate comparative bovine proteome databases for liver, kidney, muscle, plasma and red blood cells can now be routinely employed due to the significant advances in mass spectrometry and in-line multi-dimensional capillary liquid chromatography and capillary electrophoresis.

Table 1. The frequency of occurrence of bioactive fragments in a protein chain calculated for a variety of bovine muscle proteins. [Data from citation 34]

				Frequency of occurrence of bioactive fragments in a protein chain		
Protein	No in the UniProt database	Number of activities	ΣA	Highest frequency	Second highest frequency	Third highest frequency
Collagen α-2(I)	P02465	20	1.620	0.660[a]	0.210[b]	0.194[c]
Elastin	P04985	14	1.267	0.849[a]	0.149[c]	0.103[d]
Hemoglobin α	P01966	11	0.507	0.310[a]	0.106[c]	0.021[e]
Myomesin-1	P80473	15	0.473	0.291[a]	0.071[c]	0.018[b, f]
Myoglobin	P02192	8	0.468	0.331[a]	0.052[c]	0.026[e, g]
Myosin heavy chain IIb isoform	Q6XKR6	9	0.464	0.330[a]	0.047[c]	0.029[g]
Myomesin-2	Q32LP3	17	0.452	0.300[a]	0.064[c]	0.016[h]
Actin, α skeletal muscle	P68138	12	0.435	0.329[a]	0.072[c]	0.011[i]
Actin, cytoplasmic 1	P60712	12	0.406	0.330[a]	0.072[c]	0.011[i]
Troponin I, cardiac muscle	P08057	8	0.402	0.283[a]	0.052[c]	0.033[g]
Troponin T, cardiac muscle	P13789	7	0.350	0.270[a]	0.049[c]	0.030[g]
Nebulin	Q28140	5	0.349	0.235[a]	0.068[c]	0.030[g]
Desmin	O62654	8	0.330	0.251[a]	0.043[c]	0.019[j]
Vimentin	P48616	11	0.307	0.210[a]	0.047[c]	0.009[b, d, f, i]
Tropomodulin-4	Q0VC48	9	0.307	0.203[a]	0.055[c]	0.012[b]
Tropomyosin α-1 chain	Q5KR49	5	0.296	0.201[a]	0.039[c]	0.028[j]
Myosin light polypeptide 6	P60661	5	0.280	0.240[a]	0.027[c]	0.007[h, i, j]
Tropomyosin β chain	Q5KR48	5	0.268	0.183[a]	0.035[c]	0.021[g]
Troponin C, slow skeletal and cardiac muscles	P63315	4	0.260	0.236[a]	0.012[c]	0.006[h, j]

[a]Antihypertensive; [b]Antithrombotic; [c]Inhibiting dipeptidyl aminopeptidase IV; [d]Regulating mucosal membrane; [e]Antioxidant; [f]Antiamnestic; [g]Bacterial permease ligand; [h]Ion flow regulating; [i]Activating ubiquitin-dependent proteolysis; [j]Neuropeptide inhibitor.

A major driver for the industrial biotechnology industry over the past decade has been the need to access fast and efficient downstream methods for the separation of the desired product(s) from the chosen feed stock source to the desired level of purity and functional activity, whilst ensuring that process and marketing costs and other commercial parameters are kept to acceptably low levels. When the objective

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becomes the commercial application of a specific bioactive in a specific well defined physiological/ medical role, rather than as a nutraceutical component in a mixture of additives/ ingredients for use in functional or fortified food products, similar needs for efficient, high resolution methods of separation and purification arise. Availability of these technologies and associated know-how, particularly when they have as their basis a common platform, represents a significant and highly competitive advantage.

The requirement of such platform technologies, which increasingly allow automated/continuous processing, and how they can be implemented in the manufacture of a variety of different bioactive products will be emphasised as part of the next article (Part II).

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"Bioactive Choices, Business Models and Breakthrough Technologies for the Sustainable Manufacturing of High-Value Added Bioactive Products from Red Meat Sources: Part II".

INTRODUCTION: Scarcely a day now goes by when the issues of sustainability and the "green" economy within the Australian manufacturing sector is not raised in the press or in public discussion. By many international standards, Australia has the resource base and capability to deliver at global scale numerous products and commercial opportunities within this "green" economy through the application of more sustainable manufacturing practices. Why is it then that these opportunities have not been further advanced as high value-added secondary or tertiary processing and product manufacturing industries and why have the vast number of committee findings and policy frameworks – often funded within the Australian context by Commonwealth or State Governments – not gained greater traction?

Central to these considerations are the requirements that sound management, good governance, astute business models, resource availability, a skilled workforce and understanding what are the products needed and the nature of their markets globally all concurrently exist. The development of a "bioactives" industry based around value-added "bioactive" compounds and processes in Australia is a point in question.

In earlier articles of this series, several issues relevant to the growth of an Australian "bioactives" industry generated from red meat byproducts were examined. These opportunities were compared to several well documented, and internationally sourced, case studies related to nutraceutical products. Some of the characteristics of such "bioactives" industries were discussed and several exemplar approaches were examined whereby value-added "bioactive" compounds, derived from microbial, plant or animal sources, had been successfully commercialised by overseas companies. In each case, the modus operandi of these companies was to capture via an integrated supply chain approach new opportunities in renewable resource manufacturing that target human nutrition and well being. Central to these developments was the requirement and availability of "breakthrough" technologies that enabled the timely commercialisation of a specific class of products.

TECHNOLOGY REQUIREMENTS: The ability to deploy and the freedom-to-operate advanced processing technologies and the associated know-how to economically make and deliver safe and efficacious products with useful application scope is thus a recognised and essential prerequisite for an Australian "bioactives" industry. Importantly, with the current emphasis of manufacturing on sustainability, i.e. manufacturing processes that concomitantly use less energy and water and are waste and risk generation lean, yet capable of achieving greater process productivity, better resource utilisation and higher product profitability, it is not surprising that new business paradigms are also required. With appropriate levels of intensification of production through advances in process engineering, such business models for "bioactive" industries can be imbedded within different sectors of the supply chains to deliver products that (i) lead to nutritional and health benefits, (ii) require lower consumption of energy or alternatively provide new sources of energy, (iii) permit the use of significantly lower amounts or no hazardous/toxic chemicals in the production, and

(iv) permit minimisation of extensive waste disposal or land-fill compliance requirements.

Realisation of these developments and outcomes underpins the so-called 'green economy'. Although in the past, the view may have often been held that it is not easy to be green – hence the cradle-to-grave metrics (and consequences) that lead to approximately 10% (at best) of what enters the supply chain pipe exits as goods and services, the rest being waste[1], alternative cradle-to-cradle manufacturing approaches are now increasingly being championed. The impact of these developments is not being overlooked by major multi-national companies active in the food, chemical and consumer product sectors. This shift in emphasis has lead overseas to major multi-billion dollar businesses to be based around the implementation of multistage extraction and purification technologies using feedstocks derived from large scale fermentation methods or food processing waste streams, such as whey or plant/vegetable debris, coupled with advanced product quality control techniques, and in-depth scientific evaluation to validate the claimed nutritional, biological and epidemiological advantages of the derived "bioactive" compounds and materials.

Similar significant shifts in business paradigms involving new or existing product opportunities, their manufacturing processes and marketing strategies are equally relevant to "bioactive" products from the red meat industry. However, due to:

(a) the current nature of the technologies and work force skills involved,

(b) the entry points to gain access at scale to the relevant feedstocks,

(c) the commercial gains that can be achieved through supply chain integration from the producer to retailer, and

(d) the need to market high quality products globally,

realisation of these opportunities will most likely be better achieved through the strategic formation of a variety of commercial alliances of different magnitudes and impact. In previous articles, how such alliances have evolved in other areas of the "bioactives"/ nutraceutical market were summarised. Moreover, several low hanging opportunities of relatively short term horizons, derived from protein byproducts of the red meat industry, were prioritised. These valuable "bioactives" included products derived from major feed stock opportunities related to red meat(muscle), blood (plasma), bone and connective tissue. To this end, besides the commercially important plasma proteins, several enzymes and proteoglycans, a pathway to the production of various bioactive peptides with significant antihypertensive effects, for example, angiotensin I-converting enzyme (ACE) inhibitors[2], were identified (see for example Part I), whilst the utility of several bioactive proteins, peptides and other nutraceutical molecules with immuno-modulatory, hypocholesterolaemic, opioid, anti-thrombotic, anti-oxidative and antimicrobial properties obtained in yields compatible with viable large scale production procedures from the lower value off-cuts/tissues and offal of red meat animals were described.

In this Part II, the application of technologies required for the production from red meat animal sources and subsequent use of a second class of commercially significant "bioactive" products -- substances generated from lipid, fatty acid or non-polar steroidal compounds -- is

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1 Source: http://www.wri.org

2 Arihara, K. *Meat Science*, (2006) **74**, 219–229

examined. Again, in many cases these compounds represent either value added products themselves or the starting materials for the preparation of other high value added products. In addition some of the more innovative and efficient technologies that can be used in their extraction and purification, such as supercritical fluid extraction (SFE), are relatively mature technologies in terms of their application in other areas of industrial production, e.g. the use of SFE in the decaffeination of tea and coffee beans, but within the red meat industry their application has not generally reached a similar stage of development or commercial activity. This lag is only a matter of time, since increasing public and economic pressure will drive a transition towards environmentally friendly green processing technologies that avoid the use of traditional, and often toxic or hazardous, organic solvent extraction methods.

CONJUGATED LINOLEIC ACIDS (CLAs) AND OTHER LIPID PRODUCTS: The conjugated fatty acids, including the conjugated linoleic acids (CLAs), are examples of this second class of compounds where significant commercial opportunity exists, due in part to their anti-carcinogenic, anti-oxidative and weight management properties in humans. Although kangaroo meat has been ranked as the red meat source containing the highest concentration of CLAs[3], meat products from grass-fed cattle, sheep and other ruminants are excellent sources of CLAs, with 3-5 times higher levels obtained from grass feed rather than grain-fed animals[4], an observation that suggests that the microflora of the ruminant gut plays a critical role in the biosynthesis of the CLAs.

As noted above, the CLAs exhibit a diverse and important range of biological effects which directly impact on human well being. Although their precise modes of biological action(s) as functional food ingredients have still to be fully delineated[5,6], a significant body of scientific literature indicates that the CLAs have important potential for use in the treatment of several cancers, immune dysfunction including oxidative stress, atherosclerosis, other aberrations of lipid and fatty acid metabolism, obesity and diabetes and even bone formation. For example, CLAs exhibit geometric isomer-specific effects on gene expression patterns of relevance to important intestinal processes, e.g. carcinogenesis and Ca transport[7], whilst recent human clinical trials with CLAs as supplements have indicated improvement in quality of life measures of Crohn's disease patients with modulation of T cell responses and amelioration of inflammatory bowel disease. The antioxidant capacities of the CLAs, possibly functioning through modification of prostaglandin metabolism, Δ^9-desaturase activity and apoptotic pathways, would account[8] for their protective effects in several cancers, atherosclerosis, diabetes mellitus, platelet aggregation and effects on the immune system.

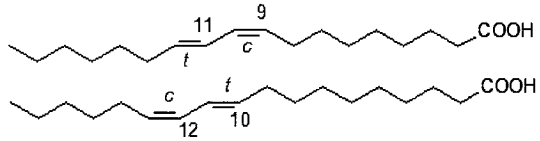

Figure 1: Structures of the abundant 'rumenic' CLAs

The name CLAs collectively includes all octadecadienoic acids with conjugated double bond structures. Hypothetically, 56 geometric isomers with different *cis* or *trans* configurations

[3] Kangaroo meat - health secret revealed. http://www.csiro.au/files/mediarelease/mr2004/kangaroofat.htm.
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could exist. CLAs from red meat sources are usually obtained as mixtures of geometric isomers, with the cis[9]-, trans[11]- and the trans[10]-, cis[12]-CLA isomers (Fig. 1) the most abundant. Because of their source, these isomers have hitherto also been given the lay name "rumenic" acids. The conjugated linoleic acids readily undergo isomerisation under alkali conditions. Various methods of analysis of the CLAs have been established, with the major methods based on gas chromatographic-mass spectrometric (GC-MS) analysis[9,10], silver ion chromatography[11], reversed phase HPLC with second derivative UV detection[12], fourier transform Raman spectroscopy[13], attenuated total reflection-Fourier transform IR (ATR-FTIR) spectroscopy[14] and ^{1}H and ^{13}C nuclear magnetic resonance[15].

A variety of technologies have historically been utilised to extract and isolate the CLAs, either as their free acids or the corresponding esters. These technologies have included adaptations of the traditional procedures based on binary or tertiary organic solvent extraction methods, e.g. with a chloroform/methanol (2:1 v/v) mixture (Folch method) or chloroform/methanol/water mixture (Bligh and Dyer method)[16,17], silver ion liquid chromatography[10], Soxhlet percolation with organic solvent mixtures or steam distillation. Each of these classical procedures has limitations either because large quantities of chlorinated or flammable solvents are required – leading to significant waste disposal and solvent hazard issues, high temperatures are employed – leading to reduced yields of the CLA products and considerable energy utilization, and significant capital investment for larger scale operations.

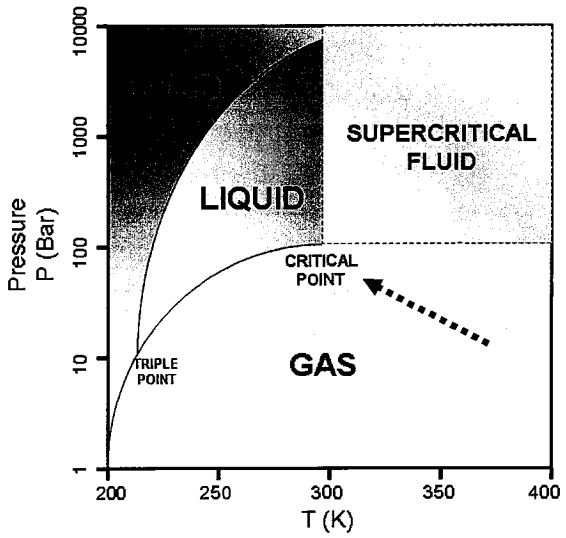

Figure 2: Schematic illustrating the general principle of supercritical fluid

During the past two decades, more benign methods of extraction of non-polar compounds from complex biological matrices have been established by taking advantage of developments in compressed gas extraction. In these circumstances, unique properties of some gases/liquids, such as carbon dioxide, water, ethane/propane/butane/isobutane, diethyl ether or some refrigerant gases like R134a, etc, can be taken of exploited. When the operational conditions of temperature and pressure transition through a critical point of the phase diagram, a so called supercritical fluid state is achieved (Fig.2). Because of the practical constraints of flammability and the need for spark-less rooms, the use of the organic based compounds is not the favoured option for product extraction, whilst the use of refrigerant compounds at

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higher temperatures has associated with some concerns related to toxicity.

For these reasons, CO_2 extraction under low temperature sub-critical operational regimes or alternatively under supercritical fluid extraction conditions represents two attractive, but not yet fully explored, options for the extraction from red meat sources of bioactives, nutraceuticals and other molecules of interest to the food and pharmaceutical industries[18], including CLAs. The option to employ CO_2 in combination with water, methanol or ethanol as a mixture can also be considered. Supercritical extraction conditions with CO_2 are achieved when the temperature is above the critical temperature of 31.1°C and when the pressure is at, or above, the critical pressure of 72.9 atm/7.39 Mpa. In this state CO_2 exhibits properties similar to those of a gas and a liquid, i.e. has a density similar to that of a liquid but with viscosity and diffusivity that are intermediate between those of a gas and a liquid. Water also becomes an excellent solvent for non-polar organic compounds when above its critical point (374°C, 218 atm) but its use is more technical challenging under these extreme conditions due to handling/safety considerations and its reactivity with steel fittings.

For quality control procedures, the determination of fat content and fatty acid composition in meat and meat products by supercritical fluid extraction is gaining popularity overseas[19,20,21]. Similar methods are also applicable for the detection and quantification of contaminants, such as dopants, anabolic sterols, drugs, chlorinated dioxins and pesticides[22,23,24,25,26], vitamins[27], nitosamines in cured meats[28], fat removal from dried meats, and documentation of fat content of meat that has been irradiated to achieve extended shelf life[29].

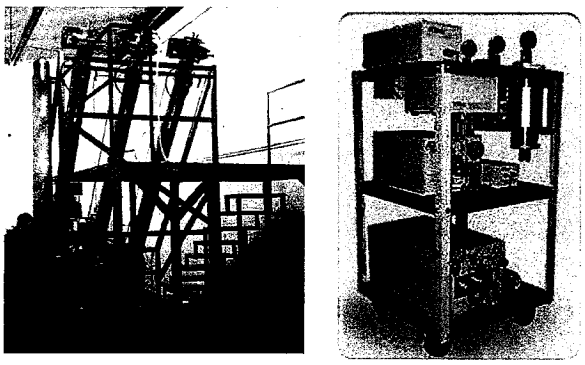

Figure 3: LHS: Recirculating Supercritical CO2 Unit, courtesy Eden Labs LLC, Columbus, OH, USA; RHS: Large laboratory skid unit, courtesy Iwoo Scientific Corp, Seoul, Korea

Supercritical fluid extraction has found broad application as process technologies in the petrochemical, pharmaceutical, fragrance, food and plant extract industries. A variety of vendors, including Phasex Corp., Thar Corp., Eden Labs LLC, Iwoo Scientific Corp. and many others, now offer large laboratory, pilot and industrial scale equipment as modular or fully integrated/automated SFE systems. Fig. 3 illustrates two such examples. The combination of CO_2 with water operating under subcritical or supercritical regimes provides access to unique extraction opportunities which satisfy the requirements of organic solvent free green, more sustainable technologies. Moreover, these options can results in superior yields and products of higher quality.

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A major driver for the use supercritical fluid extraction procedures with CO_2-water systems has been the increasing public concern about the safety and quality of products, particularly in the food and nutraceutical sectors and more stringent government mandated regulations requiring products to contain lower residual level of organic solvents[30]. Under usual operating conditions with scf CO_2, the lower operating temperatures result in mild extraction of lipids with little thermal degradation of the product. Similarly, addition of CLAs back into food products, such as the injection of conjugated linoleic acid into beef strips, can also be achieved by analogous scf techniques[31], whilst polymer impregnation with CLAs as nutraceutical additives would also appear to be a viable option by supercritical fluid technology. Because of the high solubility of fatty acid derivatives under sfc CO_2 conditions, the possibility also exist for the extraction of these compounds from animal skins with very high extraction efficiencies.

Similarly, scf methods have been employed to microencapsulate lipid molecules with protein carriers to enable gated release mechanisms to operate[32], whilst the extraction of cholesterol and other sterols from bovine brain and other tissues by scf CO_2 procedures has been documented[33,34]. Finally, the potential of using scf methods as an integrated part of the rending process generating fats from red meat animal waste should not be overlooked, since similar methods are well suited for the purification of fatty acid methyl esters generated as part of biodiesel and biofuels processes[35,36].

Summary: As also occurs with their protein and carbohydrate cousins, red meat processing streams provide a rich source of bioactive lipids. Their structures however dictate that alternative methods of extraction and purification have to be employed. Supercritical fluid extraction technologies satisfy many of the criteria for their production with the added bonus that the processes in principle have the potential to be environmentally more friendly, with the derived products not requiring the use of hazardous or toxic organic solvents. Experience gained in other areas of application of supercritical fluid extraction technologies in the food industry suggest that although the initial capital investment is high, the return on investment due to improved product quality and yield will be equally high. Importantly, the engineering of large scale systems has already been implemented for the manufacture of many other types of products, ranging from fragrances, essential oils, extraction of herbal medicines through to deodorising oils and fats, so there is a large knowledge base available that could be transferred to the production of this class of bioactives from red meat sources. The challenge is not the science or its impact as more sustainable technologies, but rather the need to better integrate the various components within the supply chain into an efficient value-adding industry.

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"Bioactive Choices, Business Models and Breakthrough Technologies for the Sustainable Manufacturing of High-Value Added Bioactive Products from Red Meat Sources: Part III".

Background: What are the hallmarks of a commercially successful red meat bioactive? Certainly, the key requirements of market need; product quality, safety and efficacy; brand uniqueness; and competitive manufacturing costs are essential features. However, other issues such as the bioactive's natural abundance, stability, suitability for scalability in manufacture, the number of unit operations needed to achieve optimal production yield, level of technical and financial risk, product utility in terms of mass-based performance; breadth of application; location of the product within a broader supply chain utilisation, and many other process-specific, manufacture-specific and product-specific attributes all coalesce to determine whether the effort to commercialise a specific red meat bioactive is warranted. Based on the precedents of other value-adding industries, all of these requirements can be objectively addressed through rationale choice of the bioactive, linked to the appropriate business model and deployment of innovative technologies.

Due to the advanced quality control procedures, relative disease free environment and strict biosecurity practices of the Australian red meat industry, the stage has been reached for a more comprehensive spectrum of bioactive product outcomes to be realised by capturing, in technical and commercial terms, the multiple opportunities now offered from the development of more efficient technologies over the past decade that impact across the product supple chains. This progress underpins the emergence of more advanced, yet commercially viable, technical opportunities and solutions - developments that resonate with the foresight provided by the participants who attended a Meat Industry Co-Product Biologicals Workshop in November 2001[1] and subsequent Workshops sponsored by the Meat and Livestock Australia (MLA).

The value proposition: Simply stated, the growth in scientific know-how, greater market awareness and the impact of new ways to achieve process automation and optimisation underpin the value proposition. The case can be re-emphasised that the Australian red meat industry could achieve an increasingly significant leadership position globally in terms of potential product offerings and become a pathfinder for the industrial deployment internationally of highly advanced manufacturing technologies related to the production of red meat bioactives. Implementation of these outcomes could occur at a time when other industries, further along the supply chain nationally and internationally, are showing renewed interest and greater need for products obtained from bio-based manufacturing industries. In particular, the impact of Australian-made bioactives on the emerging nutriceutical, functional additive/supplement, secondary and tertiary processing industries in the food, chemical and consumer product sectors could be considerable, provided both capacity and capability are integrated with business acumen. Even a cursory inspection of trends overseas in other areas pertinent to value-added products obtained from agricultural sources, such as the strong commitment shown in recent years by the horticultural/plant and dairy industries and their research providers in the Netherlands, elsewhere in Europe, Asia and other Northern hemisphere countries, to generate new product or improved processes based around the vision set out in various sector specific "Biorefinery Strategic Plans" e.g. the European Biorefinery Joint Strategic Research

[1] (a) Co-products Newsletter. http://www.redmeatinnovation.com.au/.../12-fifth-quarter-march-2002.pdf. (b) MLA Workshop Reports: 'Realising the potential' 25 November 2005; 'Evaluating our opportunities', 1-2 June 2006; 'Bioactives –Bioprocessing cost estimation', 19-20 April, 2007; 'Seizing the Opportunity', 9-10 October 2009; and 'We can do this', 11-13 October 2011.

Roadmap[2], leads to the realisation that a significant paradigm shift is underway in other counties in terms of business investment, staff training and acquisition of manufacturing skills and methods, and product definition and offerings across these fields.

Because of its very efficient primary production practices and high abundance levels of natural raw materials, in Australia the lack of suitable animal-derived feed stocks – blood, other biological fluids or tissues --- or the lack of collection logistics do not represent a constraint for these Australian-based opportunities. Supported by very high levels of animal husbandry and progressive quarantine management standards, Australia has, in fact, been for several decades a major supplier of some specialised red-meat derived bio-products, e.g. FCS and other blood derived processed products, to overseas markets. In terms of red meat-sourced raw materials, Australia certainly has the capacity to deliver at global scale, numerous additional commercially viable products through the application of scalable manufacturing practices based on the available technical expertise and engineering competences. Limitations in investment capital within Australia are certainly an impediment to progress, but again a variety of options exist, involving for example public-private partnerships or industry-led co-operative consortia, to drive financially sound investment strategies at the early stages of product development and commercialisation, and to alleviate risk management constraints during the more substantial stages of plant investment in a manner similar to that which has successfully occurred in other sectors of the value-adding sectors of the food manufacturing/processing industries.

From several practical perspectives, during the last decade the Australian red meat industry has not been fully in a position to commercially pursue a broad range of red meat based bioactive product opportunities as part of an industry-wide integrated development, production and operational raison d'etre, and thus has not been able to take full advantage of the paradigm shifts, captured through application of the "more-from-less" principle, that have occurred within other industry sectors dealing with alternative types of bioactives, e.g. plant-derived products that have found their way into over-the-counter nutriceuticals or even pharmaceutical products. The key to achieving similar developments within a red meat-based Australian "bioactives" industry will be the ability to simultaneously realise production intensification, cost control and process scale irrespective of the molecular nature of the target bioactive(s). Essential prerequisites for this goal to be realised are access to advanced processing technologies, deployment of associated technical know-how, a skill work force and sound market knowledge on product scope and trends. In this manner, a pipeline of safe and useful bioactive products can be efficiently delivered at commercially viable scales to markets nationally and globally.

In earlier articles of this series, several issues relevant to how the current Australian red meat bioactives industry could be strengthened were outlined. Firstly, in Part I, the inter-relationships between product choices, process design, manufacturing options and business paradigms were summarised in the context of existing and emerging technologies. Several 'low hanging' product opportunities and, alternatively, process improvements and technologies for their more efficient recovery and purification were identified. Not unexpectedly, the major feed stock sources for the higher molecular weight bioactive exemplars were red meat (muscle), blood (plasma), bone and connective tissue. The need for adaptations to current methods to enable more efficient generation of several already commercially important products, namely bovine serum albumin, chondroitin

[2] Joint European Biorefinery Vision for 2030. http://www.star-colibri.eu/files/files/vision-web.pdf

sulphate/dermatan sulphate, immunoglobulins, prothrombin and haemoglobin (as prioritised[3] in the MLA *Bioactives Compendium*) was recognised. Further, additional commercial opportunities were also highlighted[4,5,6,7] for the large scale production from low value red meat tissue off-cuts or offal of various other bioactive proteins, peptides and other classes of nutriceutical molecules with hypocholesterolaemic, antimicrobial, immuno-modulatory, anti-thrombotic and anti-oxidative properties and functionalities, including cardiovascular activities, such as the angiotensin I-converting enzyme (ACE) inhibitors[8,9]. Included in this group are a diverse range of consumer product options, which have already gained approval for human consumption overseas, with the anti-oxidative histidyl dipeptides, carnosine (β-alanyl-L-histidine) or anserine (N-β-alanyl-1-methyl-L-histidine) or the anti-cholestero-genic and anti-apoptotic L-carnitine (β-hydroxy-γ-trimethyl-aminobutyric acid) being representative exemplars.

In Part II, the potential for direct impact on human well-being of a second class of commercially significant bioactive products -- substances generated from lipid, fatty acid or non-polar steroidal compounds – and the technologies required for their production from red meat animal sources were examined. Thus, some of the technical options and avenues to manufacture conjugated linoleinic acids (CLAs) with anti-carcinogenic, anti-oxidative and weight management properties in humans were considered. As noted, these CLAs exhibit a diverse range of biological effects with a significant body of scientific literature indicating important roles in the treatment of several cancers, aberrations in lipid and fatty acid metabolism, obesity, diabetes atherosclerosis, Crohn's and inflammatory bowel disease and oxidative stress and, through their effect on calcium transport, bone remodelling[10,11,12]. Although 56 geometric isomers of octadecadienoic acid with different cis- or trans- configurations are theoretically possible, the most abundant CLAs from red meat sources are the cis^{9}-, trans^{11}- and the trans^{10}-, cis^{12}-CLA isomers and are usually obtained as mixtures. Enzymatic modification and controlled alkali-mediated isomerisation represent viable technologies for the inter-conversion and resolution of these isomers in order to obtain single isomer products with enhanced specific activities and greater biological selectivities.

A number of these protein- and lipid-based bioactives are now marketed overseas and increasingly in Australia, and represent multi-million dollar per annum businesses as nutraceutical products or functional food additives that can be employed[13,14,15,16], for example, as beverage products for 'elite athletes' or as nutritional supplements for the elderly with the objective in the latter case to target prevention rather than treatment of a disorder. Because the time lines to achieve regulatory approvals with such bioactive products are generally much shorter and the costs much lower compared to possible medical or veterinary uses, their applications as functional food ingredients represent low hanging opportunities, provided the bioactive can be readily liberated from latent, highly abundant and readily obtained macromolecular precursors.

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3 MLA Bioactives Compendium. http://www.redmeatinnovation.com.au/project-reports/report-categories/co-products/bioactive-opportunities-for-the-Australian-red-meat-industry, (2011) ISBN 9781741915457, 1-27.

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In the case of protein-derived bioactives, these criteria can typically be achieved by enzymatic hydrolysis of low value red meat tissue off-cuts and offal or similarly derived biological materials, such as bovine blood as a source of the globin/haemoglobin pool. In an analogous fashion, connective tissue sources provide an avenue via enzymatic processing to chondroitin- and dermatan sulphate-related polysaccharides[17], and waste skin/hides provide access[18] to collagen hydrolysates and human consumption-grade gelatins. The key to the generation of these bioactive peptides and their applications is the availability of the appropriate enzyme or cocktail of enzymes, with the specificity, abundance and purity suitable for the process task. As such, these enzymes, many of which are relatively abundant serine proteases, thioproteases, endo- and exo-peptidases or oxido-reductases in red-meat tissues, find an essential role as processing/manufacturing aids, and represent in their own right commercially valuable products. Additional, a broad range of these enzymes have application in enzyme-based diagnostic kits, in other areas of food processing, and in the manufacture of a diverse portfolio of consumer products, ranging from leather goods to specialty skin lotions. Access to appropriate and scalable technology options that enable the extraction and purification of these molecules are thus essential.

Molecular Solubility and Aqueous Two Phase Extraction of Red Meat Protein Bioactives: In this Part III, recent developments in the application of technologies for the production of enzymes and other bioactive products from red meat sources and their subsequent use has been examined from the perspective of the molecule's solubility and its ability to be partitioned between two immiscible phases. These features are fundamental properties of all molecules and have been the subject of intensive research for many decades. Knowledge of these properties underpins the large scale separation and purification of many proteins, based on precipitation, crystallisation, aqueous two phase extraction (ATPE) and related technologies. Because molecular solubility and aqueous two phase extractability are intimately related through their common physicochemical basis, the emphasis of this article has been focused on recent developments in the ATPE of proteins/enzymes. What sets recent advances apart from earlier developments and procedures in this field has been the introduction of automation, greater utilisation of continuous processing techniques, the application of robotic high-through-put methods for screening suitable conditions, and the development of more benign procedures that enable greater separation/extraction efficiencies, better recovery of bioactivities and thus higher yields per unit operation.

Compared to the conventional types of extraction or precipitation procedures based on organic solvent-water mixtures, such as the Cohn ethanol fractionation method with plasma (blood) proteins, or the Folch (chloroform/methanol (2:1 v/v) mixture) or Bligh and Dyer (chloroform/methanol/water mixture) methods[19,20] or Soxhlet percolation procedures with organic solvent mixtures for the recovery of CLAs, most aqueous two phase extraction systems (ATPEs) can be considered as much more "green", i.e. solvent free, technologies of lower environmental impact.

As historically developed, ATPEs are biphasic systems formed from two water soluble polymers. Two discrete phases can be generated from these two components by the appropriate selection of temperature, pH or the ionic strength of additives. Phase separation commences when the concentration (weight percentage) of one of the polymers (or additives) in the aqueous phase exceeds a limiting value – the set point. Figure 1 illustrates the general principles of the phase

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diagram of an ATPE system. Similar processes can occur when a water soluble polymer is mixed with a suitable kosmotropic (water-stabilising) salt or alternatively with an ionic liquid (a so-called "molten" salt) such as cholinium cations and simple anions, like citrate, lactate or acetate[21,22]. The general shape of the phase diagram with ATPE systems bears remarkable similarity to the solubility diagram of substances, e.g. proteins, in the presence of salt, e.g. ammonium or sodium sulphate, or solvent, e.g. ethanol, mixtures of different weight percentages – a feature that is not unexpected due to the commonality of their underlying thermodynamic and extra-thermodynamic properties. Partition of a biological macromolecule (bioactive) between the two phases can be described by a unique partition coefficient, K. With high throughput robotic microfluidic equipment, K values can now be obtained for any ATPE system that can be contemplated, rapidly, precisely and with minimal resource usages. This knowledge is important for process optimisation.

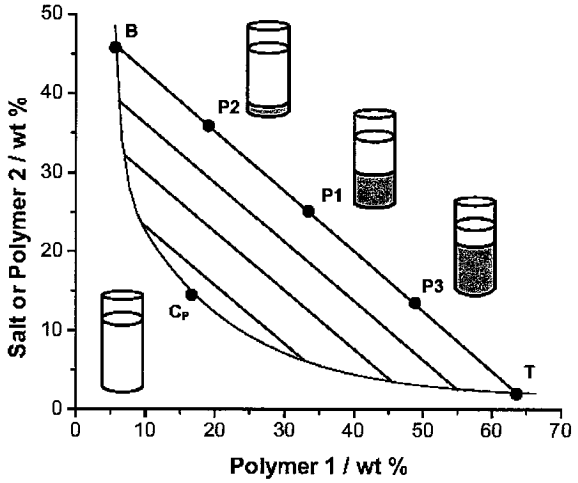

Figure 1: Schematic representation of the phase diagram for an aqueous two phase system containing two water soluble polymers or a water soluble polymer, such as polyethylene glycol, and a kosmotropic salt.

Besides the influence of the system temperature, pH and ionic strength of the added salt(s), many other parameters -- (a) the target protein's molecular weight, charge, surface properties and hydrophobicity; (b) the polymer's molecular weight; (c) the phase composition; (d) the kosmotropic characteristics of the salt, or (e) whether a biomimetic, e.g. a triazine dye, a chelating compound or a mixed mode ligand, or a biospecific affinity ligand, such as an enzyme inhibitor, like p-aminobenzamidine for bovine trypsin, has been coupled to the polymer – can greatly affect extraction efficiency with ATPE systems in a condition-dependent manner. For example, when a hydrophobic ligand such as palmitic acid is bound to PEG6000, a significant increase in affinity occurs for the extraction of α-lactalbumin but not β-lactoglobin in the presence of calcium ions, thus permitting much more efficient counter-current distribution in aqueous two phase systems[23].

Because of its versatility, ATPE procedures can be employed for a variety of bioseparation tasks, ranging from the separation of water soluble molecules through to particulates, such as cells and cell organelles, with recovery general good due to the benign chemical nature of the non-denaturing separation media. ATPE methods based on polyethylene glycol (PEG) - dextran mixtures, pioneered in the 1950's by Per Albertsson[24], have been the most commonly investigated, whilst a large variety of alternative systems based, for example, on PEG-citrate or PEG carbonate mixtures have found their way into specific applications for protein purification[25]. Because of their chemical properties and structures, polar/hydrophilic proteins tend to readily interact with PEGs, and depending on the partition coefficient values distribute between the two phase (often in the PEG phase). Collectively, these technologies have utility for the extraction of proteins from crude biological extracts, including the industrial scale production and purification of enzymes in both the batch and continuous modes.

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25 Hatti-Kaul, R. Editor (2010) Aqueous Two-phase Systems Methods and protocols:, Humana Press Inc. New York, USA, pp 1-468; ISBN: 1617370673 http://www.fishpond.com.au/Books/Aqueous-Two-phase-Systems.

Besides these polymer/polymer and polymer/salt ATPE systems, the use of thermoresponsive polymer-based phase systems and detergent-based phase systems are also attracting interest[25,26], thus enabling the dimensionality of two phase systems to be extended to three and higher order phase systems for specific applications for enzyme purification.

Moreover, to circumvent the high cost of highly purified dextrans in industrial process, the use of hydroxypropyl starch derivatives or high ionic strength salt solutions have increasingly gained favour. Besides the use of these compounds to generate the aqueous two phase system, a number of important recent developments have turned ATPE technology into a much more viable technology for large scale production of proteins, particularly enzymes. Included amongst these developments has been (i) the validation of new optimisation algorithms[27] to permit enhanced flow-sheets to be used for protein purification; (ii) application of design-of-experiment/central composition response surface methodologies[28], the introduction of robust biomimetic and biospecific affinity interaction modes of ATPE to enhance extraction selectivity[29,30], and the deployment of high throughput robotic microfluidic methods to guide the selection of optimal conditions and to obtain critical physicochemical data on solubility, recoveries and functionality of the target protein. For example, recent applications of such optimisation routines not only has permitted improved frameworks to be employed for the design of specific unit operation flow sheets and derivation of essential thermodynamic data, but have also enabled cost-performance factors to be assessed for one-stage and two-stage flow-sheets with and without the recycling of phase components.

In this manner, the economic and environmental sustainability of aqueous two-phase extraction processes can be much more rigorously evaluated[31,32,33]; new opportunities explored, including those based on the use of simplified magnetic particle technology in conjunction with ATPE methods[34,35], and the use of non-conventional ATPE systems of low environmental impact derived from novel salt systems to be validated with, for example, enzyme zymogens obtained from bovine and other tissue, such as the separation of trypsinogen and chymotrypsinogen from bovine pancreas[36]. As a consequence, new process intensified capabilities are emerging to complement the traditional ATPE methods for the recovery of transferrin, serum albumin, immunoglobulins and haemoglobin from bovine blood fractions, lactate dehydrogenase, myokinase, pyruvate kinase and malate dehydrogenase from muscle, pepsinogen from stomach or superoxide dismutase and alkaline phosphatase from liver[37,38,39,40,41,42]. Besides their important uses in diagnostic kits and some fields of chemical synthesis, broader utility is found for bovine enzymes in meat tenderisation, leather industry application and various areas of food processing as noted above.

Moreover, recent demonstrations from the biotechnology/biopharmaceutical industry have shown that systems with operational scales of between 50,000–100,000 L are feasible[43] for some APTE

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applications, whilst cost factors and productivity have been reported to be very competitive under appropriately chosen conditions. Although polyethylene glycol (PEG) itself is generally considered to be non-toxic and biodegradable, the disposal of large quantities of salt and other additives from large scale batch operations is undesirable. Re-use of the PEG over multiple cycles has been found to not significantly affect the partition behaviour of proteins, but the recovery may drop slightly. If very large-scale aqueous two-phase extraction procedures are a necessity, due to the nature or abundance of the bioactive molecule, advantage can be taken of continuous operation either as a single stage or multi-stage continuous extraction, with recycling of phase components using appropriately configured liquid–liquid extractors, thus avoiding the type of scale-up limitations that are often experienced with chromatographic separations and also allowing waste disposal problems to be minimised. Moreover, to optimise the hydrodynamics and mass transfer characteristics of the ATPE system a variety of novel engineering approaches have been developed, e.g. continuous perforated rotating disk contactors, whilst a variety of strategies[44] have been proposed to reduce energy consumption, whilst still maintaining process efficiencies.

If large scale batch extraction methods have to be utilised, then it is imperative that well designed mixer-settler tanks of high cross-sectional interfacial area are employed, permitting high mass transfer coefficients for the biomolecules and avoidance of air bubble entrapment or foam formation during transfer or mixing. Moreover, for successful large scale batch ATPE it is also important that phase separation can readily occur in the settler tank with the different ATPE streams easily collected. These technical constraints can usually be circumvented when the operation is carried out in the continuous mode. To enhance phase de-mixing in ATPE systems, the use of acoustic, electrokinetic, microwave and magnetic methods have all been examined with various levels of success[45,46]. A key area of further development of ATPE procedures will almost certainly involve the discovery of cost-effective smart (stimuli-responsive) polymers, which can selectively respond over a narrow range of the phase diagram to a change in the local micro-environment caused by e.g. a shift in the pH, temperature, electric or magnetic field, etc. Realisation of this possibility, which will be manifested as changes the conformational structure and a phase switch of the polymer from hydrophilic to more hydrophobic characteristics or vice versa, is expected to lead to more selective separation and recovery of the bioactive molecule with the polymer migrating back to the opposite phase.

How the platform ATPE technologies described above in Part III and the other technologies discussed in Parts I and II, such as chromatographic and supercritical fluid extraction procedures can be implemented at a process scale and where technically feasible operated as automated/continuous processing systems through the application of knowledge based expert systems, optimisation routines and algorithms, such as Design-of-Experiment (DoE) methods, and how the use of single-use/single path batch modes versus integrated multi-stage closed loop continuous procedures differentiates the manufacture approaches and overall productivity for a variety of different bioactive products of relevance to the red meat industry will be emphasised in the next article, Part IV.

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"Bioactive Choices, Business Models and Breakthrough Technologies for the Sustainable Manufacturing of High-Value Added Bioactive Products from Red Meat Sources: Part IV".

Background: In earlier articles of this series (Parts I-III), several pertinent considerations were brought forward to enable further examination of the business and technical cases for growth of an Australian-based red meat 'bioactives' industry. Several practical opportunities were also identified. Amongst other considerations, the question was raised not whether such an industry is technically feasible – which it is – but whether in the current economic climate within the Australian context will such an industry be sufficiently sustainable and efficient to be economically viable.

One of the fundamental tenets that drives and to some extent over-rides all of these considerations in identifying the hallmarks of a commercially successful red meat bioactives industry related to the following key issue. Simply stated, are the various organisations within the value-adding supply chain -- from the producers of the raw material from which bioactive products can be obtained through to the final stage of bioactive product retail marketing – nimble enough and able to be part of an advanced knowledge-based and integrated manufacturing industry – characterised in recently published reports from the Commonwealth Government as so-called "industries of the future"[1,2] -- and thus capture all of the advantages and commercial benefits derived from the paradigm shifts in innovation? Alternatively, is the red meat industry at large reconciled to continue an industry predominately based on the sale of red meat as consumer food products, where margins are low and international competition is becoming increasingly fierce?

If the first scenario is the target then the high cost of labour within Australia, a high value of the Australian dollar compared to other currencies or the initial CAPEX costs should not remain a deterrent, since significant gains in productivity, product differentiation based on product quality, more efficient utilisation of the substantial qualities of safe feed-stock raw materials, such as low value red meat(muscle) off cuts and offal, blood (plasma), bone and connective tissue, and the gains from the adoption of internationally best practice technologies all pull in the same direction to encourage growth in economic value from the development of new products and markets.

If, on the other hand, the second scenario continues to be pursued, then the full scale and scope of value-adding opportunities will not be realised and this will result in continuation of the practice of sending the so-called red meat waste to rendering facilities. As such the full value of the supply chain does not get captured. To grasp the opportunities that are now available though innovation, a relatively straight forward supply chain transition and integration is required based on an ability to think

1 Prime Minister's Manufacturing Taskforce: Report of the Non-government Members. Smarter manufacturing for a smarter Australia. http://www.innovation.gov.au/industry/manufacturing/taskforce/pages/default.aspx.

2 Industry Innovation Council, Trends in Manufacturing to 2020. http://www.innovation.gov.au/Industry/FutureManufacturing/FMIIC/Pages/2020Manufacturing.aspx

and act outside historical business models and capture the benefits of the so-called "Cradle-to-Cradle" approach as discussed in Part I.

To highlight these opportunities further, consider the following two examples. The first is related to the use of bovine haemoglobin selected from the *MLA Bioactives Compendium*[3], a comprehensive set of bioactive product opportunities, prepared by MLA to assist red meat processors and value-adders to select bioactive candidates with potentially large global markets services from Australian-based businesses. The second is related to products that have utility as functional food ingredients derived from low value red meat tissue off-cuts or offal through a combination of bio-catalytic, extraction and spray drying technologies. Numerous other 'case studies' can be considered based on the 5, 25 or 250 exemplars detailed as opportunities within the *MLA Bioactives Compendium* and related documents that can be down-loaded from the MLA website.

Example 1: Bovine Haemoglobin: Bovine haemoglobin, the major iron containing oxygen transport protein, has a molecular weight of ~64,500 and is one of the most abundant bioactives present in blood at the level of ~150 g/L (i.e. constitutes ~1/3 of the total erythrocyte weight) depending on the animal haemocrit levels, nutrition and health. Because bovine haemoglobin is initially sequestered within erythrocytes, which constitute ~ 95% of the cell mass within blood, this bioactive can be more readily obtained and purified at large process levels than most other types of bioactive molecules – the other plasma proteins serum albumin and gamma-globulin being the exception.

Besides its numerous other applications in the processed food industries, the pigments industry, in the consumer goods processing industries and in some areas of the chemical processing industry, the development of very high value end applications for purified cross-linked bovine haemoglobin as vesicles[4] or in other formats in medical devices as a synthetic haemoglobin-based oxygen carrier (HBOCs) has attracted considerable interest for some years for clinical use, with products currently available in the United States and European Union for veterinary use, and in South Africa for human use.[5] Although HBOCs have yet to prove to be the panacea initially proposed for replacement of allogenic blood transfusions, the potential of HBOCs has nevertheless been recognised for use with critically ill, blood depleted trauma patients in emergency settings until a transfusion is available or regeneration of red cell densities can occur.

Like all injectable products used for human therapy, stringent Phase I through Phase III trials as well as comprehensive toxicology studies are required as part of regulatory approval for use. With bovine derived products, this now includes highly sensitive screening methods to validate the absence of transmittable spongiform encephalitis contamination prior to human use as a therapeutic grade material. The cost of these activities, their time duration and the need to establish GMP-certified

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3 http://www.redmeatinnovation.com.au/project-reports/report-categories/co-products/bioactive-opportunities-for-the-australian-red-meat-industry, (2011) ISBN 9781741915457, 1-27.

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manufacturing facilities represent major considerations behind opting for this very high value end of the applications spectrum. In common with all human therapeutic products, whilst the potential rewards can be very high and the margins considerable, the risks can become equally high if any doubts arise with regulatory authorities or the public with regard to product consistency, efficacy and safety. Where access to modern advanced process analytical technologies (PATs) exists, these risks can be significantly reduced, particularly, when the manufacturing processes have been optimised and defined by modern process engineering/process design space procedures now often called Quality-by-Design (QbD) and Design-of-Experiment (DoE) strategies and concepts.

What then are the circumstances that potentially could prevail with the less stringently regulated end of the spectrum of applications for bovine haemoglobin? A variety of application uses can be contemplated here which still fall within the high value added range of multipliers when compared to the traditional markets for red meat products in the food industry. One such example relates to the application of bovine haemoglobin in gas filtration devices or life support systems involving the use of compressed air in hazardous environments, such as those found within enclosed tanks or underground mines, where the air must be free of dust and other solid particles, or toxic components, and the air quality must meet stringent regulatory standards established for human respiration, such as BS/EN 12021:1999. Frequently, in these cases ultra-high efficiency in-line filters are required compatible with use at a relative air humidity between 25-75% at atmospheric pressure and within the temperature range of 15-25°C to capture any oil aerosols generated from the compressor and remove other particles and gaseous impurities, especially carbon monoxide, with an efficiency in excess of 99.9999% (at 0.01 m). It is well known that carbon monoxide, even at 2%, i.e. ~10 ppm, is possibly the most insidious contaminant faced by users of compressed air leading to psychomotor dysfunctions, but has an affinity 300 times greater for the haemoglobin molecule than does oxygen. Therein lays a product opportunity.

The technical feasibility to manufacture bovine haemoglobin for immobilisation onto cellulosic or other support material filters for use in the final stage of stripping carbon monoxide from compressed air, under humidity and temperature conditions that are not compatible with the treatment with catalysts such as hopcolite, exists whilst the economic modelling of suitable processes can be based on established engineering principles and approaches for scale up[6]. Bovine haemoglobin can be readily isolated in very high purity from abundant bovine blood sources by a variety of separation methods, e.g. by tangential flow or dead-end membrane filtration[7,8,9] , anion exchange chromatographic techniques[10,11], metal ion affinity methods[12] and

[6] See for example: MLA Workshop Reports: 'Realising the potential' 25 November 2005; 'Evaluating our opportunities', 1-2 June 2006; 'Bioactives –Bioprocessing cost estimation', 19-20 April, 2007; 'Seizing the Opportunity', 9-10 October 2009; and 'We can do this', 11-13 October 2011.

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[11] Sun, G.; and Palmer, A.F. J. Chromatogr. B, (2008) **867**, 1-7.

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technologies for immobilisation of globular proteins are well documented[13,14]. The know-how to manufacture at scale such types of functionalised filters or adsorbents -- or for that matter other bovine haemoglobin derived products -- clearly could be established within Australia, assuming that the supply chain from animal producer, through processor/ manufacturer to the market place is appropriately integrated.

Interestingly, bovine haemoglobin can be readily converted to the corresponding haem or the compound lacking the Fe metal ion, protopophyrin IX. Because of their unique structural properties, these compounds can also be employed to generate a variety of products including selective adsorbents when immobilised onto support materials and membranes. Moreover, a range of other applications can be identified ranging from the capture of toxic metal ions from process liquid waste streams, chromatographic analysis, light capturing reagents and devices, to pigments. Again, process intensification represents important steps in the optimisation of the production and purification of bovine haemoglobin, or the derived haem and protoporphyrin IX, to the required quality levels and in the required yields. In this context, the use of procedures based on Quality-by-Design (QbD) and Design-of-Experiment (DoE) strategies and concepts can make very significant contributions to the development of efficient, more sustainable manufacturing practices at operational scales that enable improved economical benefit and enhanced productivity.

Example 2: Functional food ingredients from low value red meat tissue off-cuts or offal through a combination of bio-catalytic processing and spray drying technologies. Enzymatic hydrolysis of readily available muscle proteins results in the generation of bioactive peptides with significant antihypertensive effects as angiotensin I-converting enzyme (ACE) inhibitors[15,16]. From other tissue sources, such as the lower value off-cuts and offal, similar biocatalylic processes can be used to generate[17,18,19,20] under conditions compatible with well engineered large scale industrial production procedures and in good yields other the bioactive peptides with hypocholesterolaemic, immuno-modulatory, anti-oxidative, anti-thrombotic, anti-microbial and opioid properties[21]. Many of the proteolytic enzymes, such as the serine protease trypsin, chymotrypsin or elastase, whilst other enzymes, such as lingual lipase, carbonic anhydrase, superoxide dismutase, as identified in the *MLA Bioactives Compendium*[22], can be obtained from red meat sources and used in biocatalytic processes.

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14 (a) Graham, B., Spiccia, L. and Hearn, M.T.W. J. Chromatogr. B, (2011) **879** 844-852. (b) Hearn, M.T.W. (1987) in K Mosbach (ed) Affinity Chromatography and Related Techniques, Methods in Enzymology, Academic Press, NY, 135, 102-117.

15 Arihara, K. *Meat Science*, (2006) **74**, 219–229

16 Ryan, J.T., Ross, R.P., Bolton, D., Fitzgerald, G.F. and Stanton, C., *Nutrients* 2011, **3**, 765-791.

17 Gobbetti, M., Minervini, F. and Rizzello, C. G. In Hui Y.H. (Ed.), *Handbook of food products manufacturing – Health, meat, milk, poultry, seafood, and vegetables.* Hoboken, NJ: John Wiley & Sons (2007) 489–517.

18 Nagaoka S. In: Mine Y and Shahidi F. (Eds) *Nutraceutical Proteins and Peptides in Health and Disease.* Boca Raton: Taylor and Francis, 2006: 41-67.

19 Korhonen, H., and Pihlanto, A. in Y. H. Hui (Ed.), *Handbook of food products manufacturing – Health, meat, milk, poultry, seafood, and vegetables.* Hoboken, NJ: John Wiley & Sons (2007) 5-37.

20 Hannu Korhone, H, and Pihlanto, A., *Int. Dairy J.* (2006) **16**, 945–960.

21 Sun, Q, Luo, Y., Shen, H. Li, X. and Lei, Y. Int. J. Food Sci. Technol., (2012), **47**, 148-154.

22 http://www.redmeatinnovation.com.au/project-reports/report-categories/co-products/bioactive-opportunities-for-the-australian-red-meat-industry, (2011) ISBN 9781741915457, 1-27.

In terms of their nutritional and preventative health benefits the application of these bioactives can be directed either to a specific commercial endpoints for a specific bioactive with a well defined physiological/medical role, or alternatively for use in functional or fortified food products as a mixture of additives/ingredients with nutriceutical properties. Obviously, different needs and approaches are required depending on whether the products are targeted to specific disease related applications or to food related nutritional/well being applications, i.e. the former requires the development of much more efficient, high resolution methods of extraction, separation and purification under GMP-conditions, a higher level of regulatory approvals and surveillance, and longer time to market and thus higher CAPEX and OPEX costs.

Clearly, in terms of time to market, processing scale and ease to implement the latter types of applications with the derived bioactives intended for use as mixtures in functional or fortified food products have significant advantages. For example, the products can be offered in sterilised liquid form, i.e. as components in soups, or as dry powders by taking advantage of recent advances in process engineering using biocatalytic methods and spray drying technologies optimised by quality-by-design approaches[23,24,25]. Availability of these technologies and associated know-how, particularly when they have as their basis a common technology platform, represents a significant and highly competitive advantage.

As noted in the earlier Parts I-III, the experience gained over many years in technically similar industries, such as the biopharmaceutical, chemical processing and functional food processing industries, is highly relevant to the emergence of an Australian red meat bioactive industry. For example, a major driver for the industrial biotechnology industry over the past decade has been the need to access fast and efficient processing technologies, often called platform technologies, with the chosen feed stock source whilst ensuring that the manufacturing and marketing costs and other commercial parameters are kept to acceptably low levels.

In both of the above cases – which have many features in common with the numerous other exemplars related to bioactive product opportunities identified in the *MLA Bioactives Compendium* -- what is needed is some lateral thinking of what constitutes a commercially relevant product linked to a suitably optimised manufacturing process. Key requirements of market need; product quality, safety and efficacy; brand uniqueness; and competitive manufacturing costs all require to be recognised and simultaneously satisfied. Moreover, other, more technical, issues such as the bioactive's natural abundance variability, its stability or its structural complexity set boundary conditions for the engineering feasibility for scalability in manufacture, and the number of unit operations needed to achieve optimal production yields which have to be taken into account.

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[25] Pauly, D. Fortschritt-Berichte VDI, Reihe 3: Verfahrenstechnik (2000) **639**, i-xiv, 1-167.

Further, the impact of other process-specific and product-specific attributes such as the bioactive product's location within a broader supply chain, the broadness of its application and the utility of the bioactive product in terms of its mass-based performance will determine whether the effort to commercialise a specific red meat bioactive is warranted and the level of technical and financial risk justified. Based on the precedents of other value-adding industries, all of these requirements can be objectively addressed through rationale choice of the bioactive, linked to the appropriate business model and deployment of innovative manufacturing technologies guided as appropriate by the combination of process analytical technologies and quality-by-design (QbD) methods, design-of-experiment (DoE) procedures and design space optimisation (DSO). In Part I-III examples of the use of chromatographic, CO_2-supercritical extraction and aqueous two-phase separation methods for the recovery and purification of bioactives were summarised with specific red-meat derived bioactives in mind. In the following section, the nature of the optimisation routines that can be employed to achieve process optimisation with single-stage and multi-stage unit operations is summarised.

What QbD, DoE and DSO tools are available to guide process optimisation?

Process optimisation is essential if improved productivity, profitability and CAPEX utilisation are to be realised in the manufacture of bioactives. The deployment of Quality-by-Design, design-of-experiment (DoE) or design space optimisation (DSO) procedures (terms often used as synonyms)[26,27,28,29,30,31,32,33] in the production of value-added bioactive substances from red meat sources starts with an understanding of the available processes and the characteristics and end-uses of the products. Quality-by-Design (QbD) represents the overarching objective of optimisation and requires a systematic approach to product development; design-of-experiment (DoE) or design space optimisation (DSO) represent the means by which this objective is achieved. The most optimal approach is delivered through analysis of the design space. Information acquired during process development provides important insights to validate the design space approach, thus enabling regions to be selected where local optimisation(s) has(have) been achieved under regime(s) that is(are) compatible with the overall economic efficiency and other trade-offs of the manufacturing process and product attributes.

As a starting point, much of the essential information related to the bioactive products identified in the *MLA Bioactives Compendium* can be retrieved from readily accessible literature data-bases or is resident in specialised centres of excellence within Australia, e.g. the Victorian Centre for Sustainable Chemical Manufacturing. Moreover, the use of these optimisation tools is equally relevant to both the upstream

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[31] Basu, A. and Leong, S.S.J. J. Chromatogr.A, (2012) **1223**, 64– 71.
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and the downstream stages of bioactive product processing. In the latter case, these tools can be applied *inter alia* (i) separation techniques based on e.g. precipitation, crystallisation, chromatographic or membrane-based fractionation methods, (ii) formulation or spray-drying procedures; (iiii) supercritical extraction methods or aqueous two-phase separation methods for the types of products discussed in the earlier Part I-III. Similarly, these same tools can be deployed at the upstream stages involving, for example, the use of biocatalytic processing, bioactive immobilisation or other technical aspects associated with the adjustment of feedstock composition.

To bring definition to the product and the chosen process and to enable a foundation for commercialization activities to be based on identification of the critical quality attributes, access to robust analytical platforms commonly called process analytical technologies (PATs) are also required. In the first instance, the full suite of these PAT techniques does not have to be sited within the same manufacturing plant site, since some of the experimental data can be acquired as sub-contracted tasks, to establish the framework of the standard operation procedures (SOPs). It is common practice, when evaluating a specific process, for a set of experiments to be implemented by a team of investigators in order to evaluate which process inputs have the most significant impact on the process output(s), and what target levels of these inputs should be utilised to achieve the most desirable output(s). This relationship between inputs and outputs is typically represented in terms of contour plots, as illustrated by Figure 1.

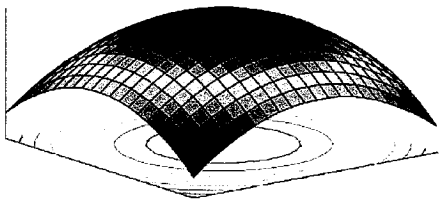

Figure 1: Response surfaces as functions of two factors having a maximum response. From reference 25

In common with many other areas of experimentation where incomplete knowledge is initially available, there will be some regions of the response contour that will be better optimised whilst some other regions which are less well optimised. Through analysis of the contour landscape, process optimisation enables the discovery of the parameter set where the most optimal outcome can be realised and aligned with actual practice carried out at different levels of scale. DoE procedures thus enable the simultaneous determination of the effect that individual and/or interactive parameters have on the outcome response and level of process robustness. In this manner, these process design tools can be used to identify which parameter settings impact most of the manufacturing efficiency and thereby enable process intensification and optimisation can be achieved. The consequence is a reduction in design (or redesign) costs, greater speed to deliver a process, reduced labour with associated manufacturing cost, better plant utilisation, power, water and other utility savings and improved material conversion to product.

In setting up a design-of-experiment routine as part of, for example, the optimisation of chromatographic process for the purification of a bovine protein, three aspects are taken into consideration[34,35]. If the fractional factorial experimental design approach pioneered by Taguchi is employed, only a very limited number of experimental

[34] Anderson, M.J. and Whitcomb, P.J., , DOE Simplified (Productivity, Inc. 2000). ISBN 1-56327-225-3.

[35] Taguchi, G., Introduction to Quality Engineering - Designing Quality Into Products and Processes (Asian Productivity Organization, 1986). ISBN 92-833-1084-5.

measurements are required to identify the optimisation "sweet spots" for this process. Overall, such a DoE approach involves or acquires knowledge on:

- The input variables which constitute overall the process, and these fall into either controllable factors such as the process volume, the type of plant that is employed, the type of the feedstock, the structural type of bioactive, etc. or uncontrollable variables linked, for example, to a catastrophic and unexpected failure of an item of equipment or a sudden loss of power due to electrical failure.
- Levels of each of the controllable variables, such as the flow rate or mixing rate, the temperature, the concentration of additives, etc. chosen to enable the process to be efficiently evaluated.
- The response landscape(s) derived for the experimental assessment of the process. Since it is desirable from practical considerations to optimise the impact of multiple variables simultaneously, rather than to optimise the process around one variable at a time in preference to another the response landscape, or landscapes should this eventuate, the design space can be interrogated to determine the factors and their levels which provide the best overall outcome for the critical-to-quality characteristics to be achieved.

Factorial design approaches are the keys to the deployment of successful DoE procedures, allowing experiments to be defined for every combination of factor levels, ranging from the most simple two factor-two level scenario to much more relevant multi-factor-multilevel circumstances where more sophisticated factorial design approaches such as the Plackett-Burmann, central composite or Box–Behnken design methods need to be employed, of which the Box–Behnken design approach with three or more levels with three or more factors is frequently favoured (cf. Figure 2). In this way, the most significant factors that affect the process output can be identified, i.e. by separating the vital few from the trivial many parameters, it is possible to capture the advantages of the well known Juran-Pareto 80:20 rules widely used in business, numerous other areas of the life and physical sciences as part of the physical/engineering optimisation of the process, as well as the economic modelling of the processes themselves. In this manner, it is feasible to achieve the most optimal process response contour, reduce process variability and enhance process robustness, whilst at the same time also balance the process trade-offs, which inevitably will arise when the process scale-up requirements, cost or product characteristics and attributes, such as stability, are to be taken into account.

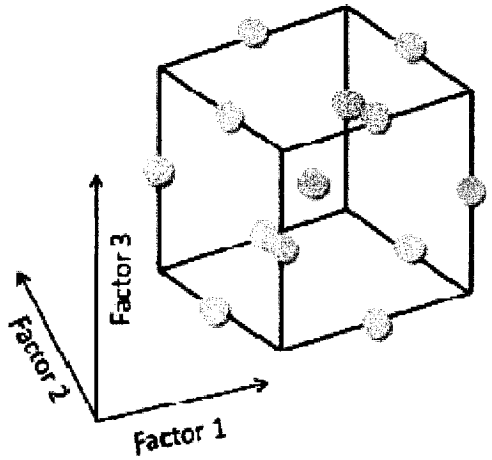

Figure 2: Example illustrating the use of the Box-Behnen design approach for a process requiring simultaneous optimisation of three (or more) critical parameters. In this example three factors are involved with each point representing a specific factor value for an individual experimental evaluation. [From reference 25].

Deployment of these tools is relevant to the more efficient manufacture of 'low hanging' product opportunities derived from red meat (muscle), blood (plasma), bone and connective tissue, such as several already commercially important products, namely bovine serum albumin, chondroitin sulphate/dermatan sulphate, immunoglobulins, prothrombin and haemoglobin (as prioritised in the MLA *Bioactives Compendium*). Similarly, scope exists for the application of these optimisation tools in the large scale production from low value red meat tissue off-cuts or offal of various other bioactive proteins, peptides and other classes of nutriceutical molecules; lipid, fatty acid or non-polar steroidal compounds, including the manufacture of conjugated linoleinic acids (CLAs) with anti-carcinogenic, anti-oxidative and weight management properties in humans, and the relatively abundant serine proteases, thioproteases, endo- and exo-peptidases and oxido-reductases present in red-meat tissues for use subsequently as processing/manufacturing aids. As evident from their successful application in allied fields of industrial biotechnology and bioprocessing manufacture, collectively, these methods offer considerable potential for processes operated in the single-use/single path batch mode or alternatively as more integrated multi-stage procedures to achieve enhancement of the overall productivity with a variety of different bioactive products of relevance to the red meat industry. Their adoption and incorporation as tools to guide the process design towards the intended endpoints is thus highly relevant to the further growth of an Australian based industry.

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