EFFECTS OF VARIOUS RICH-FIBRE EXTRACTS ON CHOLESTEROL BINDING CAPACITY DURING *IN VITRO* DIGESTION OF PORK PATTIES

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Abstract

Intake of foods containing high levels of cholesterol harms human health, and an increase in intake of dietary fibre (DF) may mitigate these negative effects. Co-products obtained from fruits juice extraction (lemon, grapefruit and pomegranate), lemon ice-cream production and tiger nuts "horchata" (beverage) have been used for the production of rich-fibre extracts used as dietary fibre sources. The purpose of this study was to examine the effect of these fibre sources additions on cholesterol retention during the *in vitro* digestion of pork patties. The control patties were prepared without fibre addition and for the rest of patties a 10% of each DF was added. The pork patties were then passed through an *in vitro* digestion model that simulated the composition of the mouth, stomach and small intestine juices. After digestion and centrifugation the product separated into 3 phases (oily, aqueous and pellet phase). The effect of each DF on phase distribution and the amount of cholesterol retained in each phase were evaluated. All DF studied provoked an increase in the cholesterol retained in the pellet phase. Pomegranate DF showed the better results (32% cholesterol retained in the pellet phase). The application of these rich-fibre extracts in food elaboration process due to their healthy properties could be very interesting if one of the most important properties that can be highlighted is their ability to adsorb cholesterol.

Introduction

The association between elevated plasma LDL-cholesterol concentration and increased risk for heart disease has made the scientific community aware of dietary sources that might effectively reduce plasma cholesterol levels. Several studies have documented that dietary fibre (DF) lowers the risk for coronary heart disease[1] by reducing the risk of type-2 diabetes, body weight, and serum low-density lipoprotein-cholesterol levels[2] and absorbing bile acids. Several DFs have been reported to interact with bile acids in the small intestine, resulting in a lower level of reabsorption and a higher level of excretion of bile acids, thus increasing the hepatic synthesis of bile acids from blood cholesterol.[3,4] However, there are no reports on the direct adsorption of cholesterol (at the jejunum) from de diet, possibly because of the difficulty in designing a model system. Incorporating fibre sources into our diet may provide a useful adjunct to a low-saturated fat diet, and may have a further beneficial effect for individuals who have mild-to-moderate hypercholesterolemia.[5,6]

Consumers by and large understand that fibre is a critical dietary component. In the 2012 International Food Information Council survey,[7] fibre ranked third, just behind caloric content and whole grains, among key components take into consideration in making purchase decisions about buying packaged foods or beverages.[8] So, there is a trend to find new sources of DF that can be used as ingredients in the food industry.[9] One of these sources is the agronomic and agroindustrial co-products, which have traditionally been undervalued. There is an increasing interest in recovering that material, which may be used among other uses, as sources of DF destined to supplemented low-in-fibre food products.[10-14]

To understand the DF bioactivity in foods, *in vitro* digestion systems appear to provide a useful alternative to animal and human models for rapidly screening food ingredients and is also ethically superior, faster and less expensive than *in vivo* techniques.[15,16] As the results of *in vitro* digestion depend upon many factors associated with food composition, structure and amount, a meat product (as source of lipid compounds) has been selected to understand the DF interactions with lipid digestion products.

Thus, the purpose of this study was to examine the effect of various rich-fibre extracts (from agroindustrial coproducts) added to pork patties, on cholesterol levels, during their *in vitro* digestion.

Materials and methods

Dietary fibre samples

DFs from different agroindustrial coproducts were obtained. Lemon dietary fibre (LDF), grapefruit dietary fibre (GDF) and pomegranate dietary fibre (PDF) was obtained from lemon (*Citrus lemon*), grapefruit (*Citrus paradisi*) and pomegranate (*Punica granatum*) juice industry coproducts, respectively. Lemon albedo dietary fibre (LADF) was obtained from lemon ice cream industry coproducts. Tiger nut dietary fibre (FIBRE) was obtained from tiger nut (*Cyperus esculentus*) milk elaboration process coproducts. The preparation of DF from these agroindustrial coproducts has already been reported by IPOA Research Group.[10,12,17,18] The milled DF sources powders were transferred to airtight plastic bags and stored in a desiccator at room temperature prior to parameters determination. Figure 1 shows all DF tested samples. Physicochemical and technological characterization of these DF has been reported by Bailina (2014).[19]

Determination of Total, Soluble and Insoluble dietary fibre content

Total (TDF) and insoluble (IDF) dietary fibre were determined following the enzymatic-gravimentric method 985.29.[20] Soluble dietary fibre (SDF) was calculated by subtracting the IDF proportion from TDF. All analyses were carried out in triplicate.

Cholesterol Adsorption Capacity of dietary fibres

The cholesterol adsorption capacity (ChAC) tries to evaluate the fibre capacity to adsorb cholesterol into its matrix, when the fibre is in contact with a simple system as water and oils mix. The ChAC of all DF samples was determined as follow. Briefly, 20 g ultrapure water and 20 g sunflower oil (added with 100 mg/kg cholesterol; control) were homogenized at 8000 rpm for 30s (IKA-Ultra-Turrax T25, Germany). Immediately, 4 g of each DF were added and the homogenization process continued until reach 2 min. The mixture was transferred into a centrifuge tube and heated in a 37ºC water bath for 1 h. After cooled down to room temperature (25ºC) the tubes were centrifuged (Sigma 3-16PK, Sigma, Maryland, EE.UU) (3000 rpm, 20 min). The supernatant was discarded and the pellet phase was used for cholesterol determination by HPLC.

The ChAC (%) was calculated as follow:

$$\text{ChAC (\%)} = \frac{mg\ cholesterol\ adsorbed\ by\ DF\ sample}{mg\ cholesterol\ control} \times 100$$

Meat patties preparation

Three independent replicates of each batch were prepared at the IPOA Research Group Pilot Plant at the Miguel Hernández University. A simple formula was used to obtain a base batter as follows: 49% lean pork meat, 49% pork backfat, 2% sodium chloride. This mixture was divided into 6 batches. A 10% of each rich-fibre extract (LDF, GDF,

PDF, LADF and TNDF) was added to 5 batches. The 6th batch was the control, without fibre addition.

To obtain the base mixture, pork trimmings were ground through an 3-mm plate (Olotinox, Olox, Spain) in a mincer attached to a mixer (CATO 114, Sabadell, Spain) and then the salt was added into the bowl and mixed with the spiral dough hook at medium speed (80 rpm) during 5 min. For each treatment, the corresponding amounts of rich-fibre extract were added and then mixed again for 5 min. The pork patties were placed in individual Albal zip-lock bags (Cofresco SAU, Madrid, Spain) and then cooked in a water bath until the core temperature reached at 75ºC. The pork patties were stored at -10ºC for experiments.

In vitro digestion model

The standardized static *in vitro* digestion method (aerobic conditions) suitable for food, reported by Minekus et al. (2014), was used in this study. The simulated digestion fluids (Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)) were prepared following the recommendations of Minekus et al. (2014).[16]

1. Oral phase: About 5 g of pork patty samples (20ºC) were mixed with 3.5 ml of SSF stock solution and minced together in a Moulinex A320R1 electric mincer (SEB SA, Bourgogne, France). 0.5 ml salivary α–amylase solution of 1500 U/ml in SSF stock solution (α–amylase from human saliva Type IX-A, 1000-3000 U/mg protein, Sigma) was added followed by 25 µl of 0.3 M $CaCl_2$ and 975 µl of water and thoroughly mixed for 2 min at 37ºC on a shacking water bath.

2. Gastric phase. 10 ml of oral bolus was mixed with 7.5 ml of SGF stock solution, 1.6 ml porcine pepsin stock solution of 25000 U/ml made up in SGF stock

solution (pepsin form porcine gastric mucosa 3200-4500 U/mg protein, Sigma), 5 µl of 0.3 M $CaCl_2$, 0.2 ml of 1M HCl to reach pH 3.0 and 0.695 µl of water. The mixture was incubated 2 h at 37ºC on a shaking water bath.

3. Intestinal phase: 20 ml of gastric chime was mixed with 11 ml if SIF stock solution, 5.0 ml of a pancreatin solution 800 U/ml made up in SIF stock solution based on trypsin activity (pancreatin from porcine pancreas, Sigma), 2.5 ml fresh bile, 40 µl of 0.3 M $CaCl_2$, 0.15 ml of 1M NaOH to reach pH 7.0 and 1.31 ml of water. The mixture was incubated 2 h at 37ºC on a shacking water bath.

At the end of the process the samples were immediately cooled at 4ºC and kept standing for 14-16h.

Phase distribution of the *in vitro* digestion products

The *in vitro* digested samples were centrifuged (4ºC) at 4000 rpm for 20 min. After that, 3 phases were identified in the centrifuged tubes:[21] oily (top layer), aqueous (middle layer) and pellet phase (lower layer). Proportion of each phase (%, v/v) was measured and the amount of cholesterol in each phase was determined by HPLC.

HPLC Cholesterol determination

Samples (1 g) were mixed with ascorbic acid (250 mg) and a solution of 10% KOH in ethanol (10 ml). After mixing, the samples were heated in a water bath at 80ºC 3 min. After cooled, hexane was added and then the tubes were capped and shaken to separate the phases. The upper phase was removed to a clean tube (this procedure was repeated twice). Solvent (hexane) was removed from the tube by drying under a stream of nitrogen. The residue was resolved in pure ethanol, filtered through a 0.45 µm Millipore filter (Millipore Corporation, Bedford, USA) and then 20 µL were injected into a

Hewlett-Packard series 1200 HPLC (Woldbronn, Alemania) according to the method described by Mazalli et al. (2006).[22] The cholesterol was eluted through a Hypersil DBS-C18, 250 mm x 4.6 mm (Thermoscientific, Fisher Scientific, Madrid, Spain) column and detected by absorbance at 210 nm. A mobile phase of acetonitrile:methanol:water (50:48.5:1.5) with an isocratic flow rate of 1 mL/min and a 20-min analysis time, was used to separate cholesterol. Identification of the cholesterol peak was done by comparison of the retention times of the sample peaks with those of the standard (cholesterol standard, Acofarma, Terrasa, Spain) and by the peak spectrum analysis. Quantification was done by external standardization, the standard curves being constructed with 6 points using solutions of the standard.

Analysis of data

Data were analyzed for significant differences using Two way ANOVA follow by Tukey post-hoc test with significance set at $P<0.05$ by statistical software IBM[®] SPSS ver. 22 for Windows (IBM, New York, USA). Data were presented as mean±SD.

Results and Discussions

Dietary Fibre content and Cholesterol Adsorption Capacity of rich-fibre extracts

Table 1 shows TDF, IDF and SDF content of tested DF sources. TDF content ranged from 44.8 to 69.1 g/100g. The highest TDF content ($P<0.05$) were found in DF from citrus juice processing (LDF and GDF). Similar results for TDF content have been reported in orange coproducts[23] and lime coproducts.[24] The lowest TDF content ($P<0.05$) was found in LADF, the other DF extract obtained from a citrus fruit. These differences in TDF between citrus fruits seem to be more dependent on the industrial source (juice extraction *vs* ice-cream production) than on the citrus species used (lemon

or grapefruit). The three DF extracts from citrus had higher (P<0.05) SDF content than IDF. TDF content in PDF was around 50 g/100g and with a ratio SDF/IDF of 1 aprox. The TDF content of TNDF was around 60 g/100g, mainly IDF (99.8% from TDF) and little SDF (0.2% from TDF). The TNDF had the highest (P<0.05) IDF/SDF ratio (496.6). This ratio was also higher that than reported for fibrous residues from other dietary fibre coproducts.[17]

For certain food applications, DF concentrates should have a balanced content of soluble and insoluble fractions; in the case of PDF the two fibre types are present at almost equal levels. Thus, it exhibits a good balance that might be important not only from the technological point of view but also from the nutritional and functional. IDF is responsible for the increased bulk of the stools and helps to regulate bowel movements. SDF play a significant role in the reduction of cholesterol level and blood pressure, prevention of gastrointestinal problems, and protection against the onset of several cancers, which include colorectal, prostate, and breast cancer.[18,25]

The *in vitro* cholesterol adsorption capacity (ChAC) of the tested DF sources is showed in Figure 2. It is important to highlight that this property has been measured directly upon the rich-fibre extract, without simulating any digestion process. PDF showed the highest ChAC (>70%; P<0.05), much higher than that the other DFs (ranged from 23 to 29%; P>0.05). In any case, all DF tested samples showed ChAC values higher than 20% which it is very interesting and promising. The results obtained for PDF related to their TDF, IDF or SDF content (Table 1) couldn't explain their high ChAC value obtained. So, it must be another reason to explain it and it could be related with the polyphenols content in PDF. Some authors have reported high content of polyphenols in pomegranate fruit and also in coproducts from pomegranate juice extraction process.[18,26] These authors[18] reported a Total Polyphenols Content of 19.30 mg gallic acid

equivalents/g sample dry weight, in PDF obtained from coproduct of juice extraction. These polyphenols may play a vital role in the suppression of cholesterol absorption by reducing solubility of cholesterol micellization or inhibiting low-density lipoprotein oxidation.[27-29] All these actions have been attributed to the highly reported interactions of polyphenols with compounds present in foods, like lipids, proteins or carbohydrates.[30]

Cholesterol distribution profiles after the *in vitro* digestion of meat patties with various rich-fibre extracts

After *in vitro* digestion of all pork patty samples, the products obtained were centrifuged to try to assess the distribution profiles of cholesterol. Figure 3 shows the proportions of each phase (oily, aqueous and pellet phase) identified after the *in vitro* digestion of all pork patties added with some rich-fibre extracts. In all samples, the highest proportion was for the aqueous phase and the lowest for the oily phase (P<0.05). The oily phase ranged from 0.57 to 1.24% without differences (P>0.05) between them. Sek et al. (2002)[21] reported that after lipids *in vitro* digestion, the phase behaviour of their lipolytic products was: an oily phase containing cholesterol and undigested triglycerides and diglycerides; an aqueous phase containing bile salt, fatty acids and monoglycerides; and a pellet phase containing approximately 5 mM of fatty acid, presumably as an insoluble soap. A number of *in vitro* studies have shown that DF can alter lipid digestion. Boisen and Eggun (1991)[31] suggested that the inhibition mechanism of most fibres might be due to the absorption of enzymes into the fibre matrix, or unspecific binding to the fibres. Lairon et al. (2007)[32] reported that the main mechanisms are that some SDF forming viscous solutions drastically reduce the rate of lipid emulsification, with a resultant noticeable lowering of the extent of fat lipolysis.

Various DF sources also can bind bile acids, as well as mixed micelle components such as monoacylglycerols and free fatty acids or free cholesterol, thus explaining the partial disruption of the micellization process leading to reduced micellar solubilisation of lipid moieties and, finally, to blunted and/or delayed intestinal uptake of lipid moieties and cholesterol.[15,32-34]

Table 2 shows the cholesterol retained (%) in each phase after the *in vitro* digestion process of meat patties with various rich-fibre extracts. As can be seen in the results for control sample (meat patty without dietary fibre), the oily phase contain all the cholesterol. When some rich-fibre extracts were added to meat patty, also some amount of cholesterol was detected in pellet phase. In any sample (control or with rich-fibre extracts) cholesterol was detected in aqueous phase. So, the effect of the rich-fibre extracts on cholesterol retention like to be related with the cholesterol retained in pellet phase. The highest ($P<0.05$) proportion of cholesterol retained in this phase was found for PDF, followed by LDF, TNDF, LADF and GDF in this order and with differences ($P<0.05$) between all them. The effect of dietary fibre likes to be mainly based on direct binding of cholesterol which cannot be reabsorbed by the body and thus are excreted. Although this mechanism of direct binding forces between fibre and cholesterol has been usually attributed to IDF, some studies indicate that also can be associated to SDF, in analogy to the cholesterol-lowering effect of IDF.[35] These authors reported that these direct binding forces could be hydrophobic interactions. However, this still controversially discussed and the detailed mechanisms remain unclear.

Looking the content in SDF and IDF of each extract (Table 1) it doesn't like to be any direct relation between their content and the amount of cholesterol retained by the rich-fibre extract (Table 2). However, the extract that showed the same proportion of SDF

and IDF (PDF) also showed the highest (P<0.05) proportion of cholesterol retained in the pellet phase.

It must be noted that the PDF extract that showed the highest (P<0.05) cholesterol absorption capacity before *in vitro* digestion (Figure 2) was also which showed the highest (P<0.05) proportion of cholesterol retained in pellet phase after *in vitro* digestion of meat patties with PDF added. There is a linear-logarithmic correlation (R^2=0.8437) between cholesterol adsorption capacity before *in vitro* digestion and cholesterol retention in pellet phase after *in vitro* digestion.

As can be seen previously discussed for ChAC of PDF extracts, the high content in polyphenolic compounds reported for these extracts also could contributed to the high levels for cholesterol retention in pellet phase.

Conclusions

The rich-fibre extracts, obtained from agroindustrial coproducts, used in this study are a good source of DF and all of them exhibit cholesterol adsorption capacity. Pomegranate dietary fibre shows the same proportion of soluble and insoluble dietary fibre and also the highest percentage of cholesterol adsorption capacity.

The addition of these rich-fibre extracts to pork patties, increases cholesterol retention in pellet phase after *in vitro* digestion. Pomegranate dietary fibre shows the highest effect. Only the content of dietary fibre or the proportion of insoluble/soluble dietary fibre in the rich-fibre extracts doesn't explain their behaviour upon cholesterol retention. Other aspects related to the type and amount of bioactive compounds (polyphenols principally) in the rich-fibre extracts (which depend on the original source) could be affecting this property.

Nevertheless, the method for determining the cholesterol adsorption capacity (simple and easy method) of dietary fibres allows a estimation to their capacity to retain cholesterol after *in vitro* digestion process (long and complicated method).

The application of these rich-fibre extracts in food elaboration process due to their healthy properties could be very interesting if one of the most important properties that can be highlighted is their ability to decrease cholesterol.

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FIGURE CAPTIONS

Fig. 1 Dietary fibres obtained from agroindustrial coproducts

(TNDF: tiger nut fibre; LADF: lemon albedo dietary fibre; PDF: pomegranate dietary fibre; GDF: grapefruit dietary fibre; LDF: lemon dietary fibre)

Fig. 2 Cholesterol Adsorption Capacity (ChAC %) of different dietary fibres obtained from agroindustrial coproducts.

(TNDF: tiger nut fibre; LADF: lemon albedo dietary fibre; PDF: pomegranate dietary fibre; GDF: grapefruit dietary fibre; LDF: lemon dietary fibre)

Fig. 3 Proportions of each phase (oily, aqueous and pellet phase) identified after the *in vitro* digestion of pork patties added with some rich-fibre extracts

(Control: without fibre added; TNDF: tiger nut fibre; LADF: lemon albedo dietary fibre; PDF: pomegranate dietary fibre; GDF: grapefruit dietary fibre; LDF: lemon dietary fibre)

Table 1. Total, Soluble and Insoluble Dietary Fibre content (TDF, SDF and IDF) of various rich-fibre extracts obtained from agroindustrial coproducts

|	LDF	GDF	PDF	LADF	TNDF
TDF (g/100g)	66.71±4.2a	69.15±5.2a	51.86±4.1c	44.80±3.5d	59.71±5.5b
IDF (g/100g)	13.51±0.9c	16.89±1.0c	24.82±1.1b	14.75±1.0c	59.59±1.4a
SDF (g/100g)	53.2±2.2a	52.26±1.9a	27.04±1.1b	30.05±1.5b	0.12±0.0c

LDF: lemon dietary fibre; GDF: grapefruit dietary fibre; PDF: pomegranate dietary fibre; LADF: lemon albedo dietary fibre; TNDF: tiger nut dietary fibre.

The results are expressed as means ± standard deviations; [a-c]Values followed by different letter in the same row are significantly different (P<0.05) according to Tukey's test

Table 2. Cholesterol retained (%) in each phase after the *In vitro* digestion process of meat patties with various rich-fibre extracts

	Cholesterol retained in each phase (%)		
	Oily phase	Aqueous phase	Pellet phase
Control	98.89±1.56a	nd	nd
LDF	75.30±5.42c	nd	18.07±1.76b
GDF	89.32±6.89b	nd	5.68±0.65e
PDF	68.14±4.19d	nd	31.86±2.19a
LADF	88.95±5.35b	nd	7.71±0.66d
TNDF	85.39±2.20b	nd	14.61±1.20c

LDF: lemon dietary fibre; GDF: grapefruit dietary fibre; PDF: pomegranate dietary fibre; LADF: lemon albedo dietary fibre; TNDF: tiger nut dietary fibre. nd: not detected

The results are expressed as means ± standard deviations. [a-e] Values followed by different letter in the same column are significantly different (P<0.05) according to Tukey's test .

Figure 1. Dietary fibres obtained from agroindustrial coproducts

(LDF: lemon dietary fibre; TNDF: tiger nut dietary fibre; PDF: pomegranate dietary fibre; LADF: lemon albedo dietary fibre; GDF: grapefruit dietary fibre)
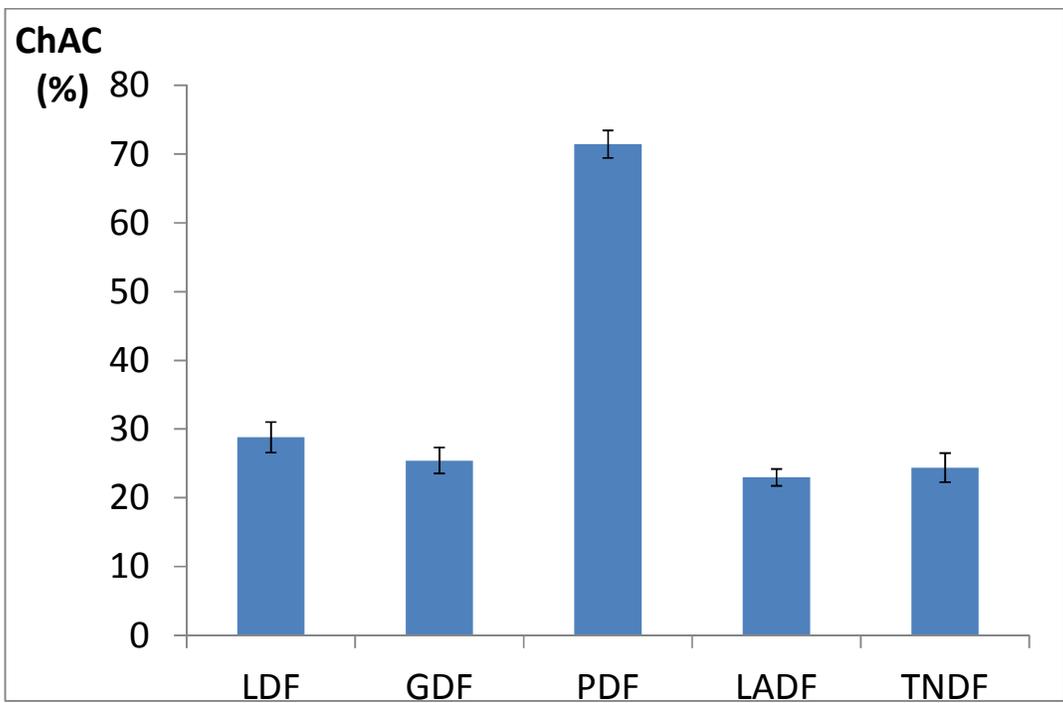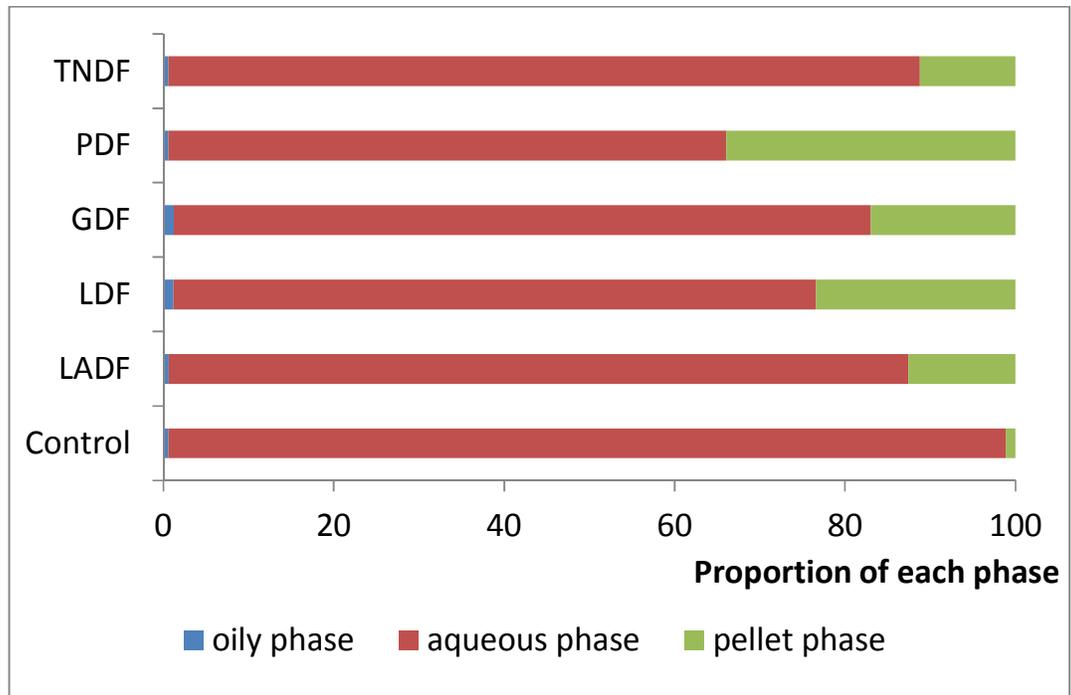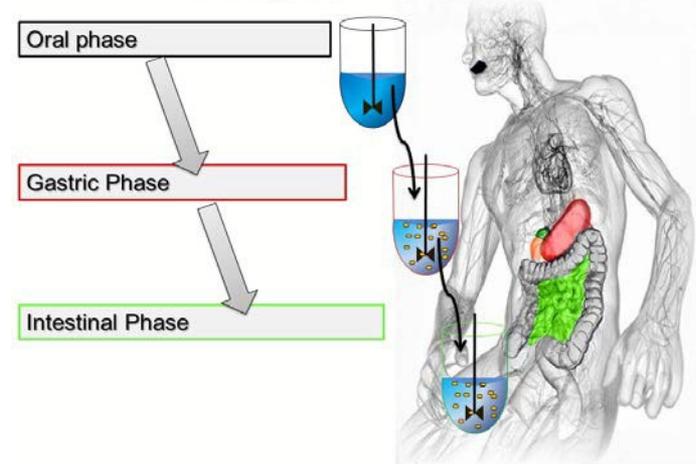
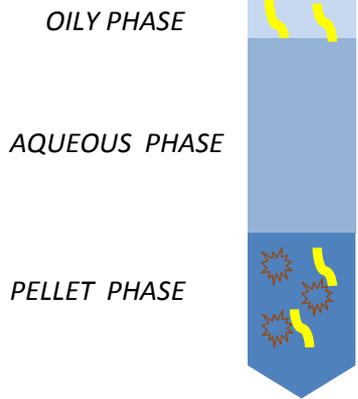DIETARY FIBRE EXTRACTS ADDED TO PORK PATTIES

DIETARY FIBRE EXTRACTS FROM AGROINDUSTRIAL COPRODUCTS

In Vitro Digestion Method for Food

Oral phase

Gastric Phase

Intestinal Phase

centrifugation

OILY PHASE

AQUEOUS PHASE

PELLET PHASE

Cholesterol retained by fibre

cholesterol

Dietary Fibre