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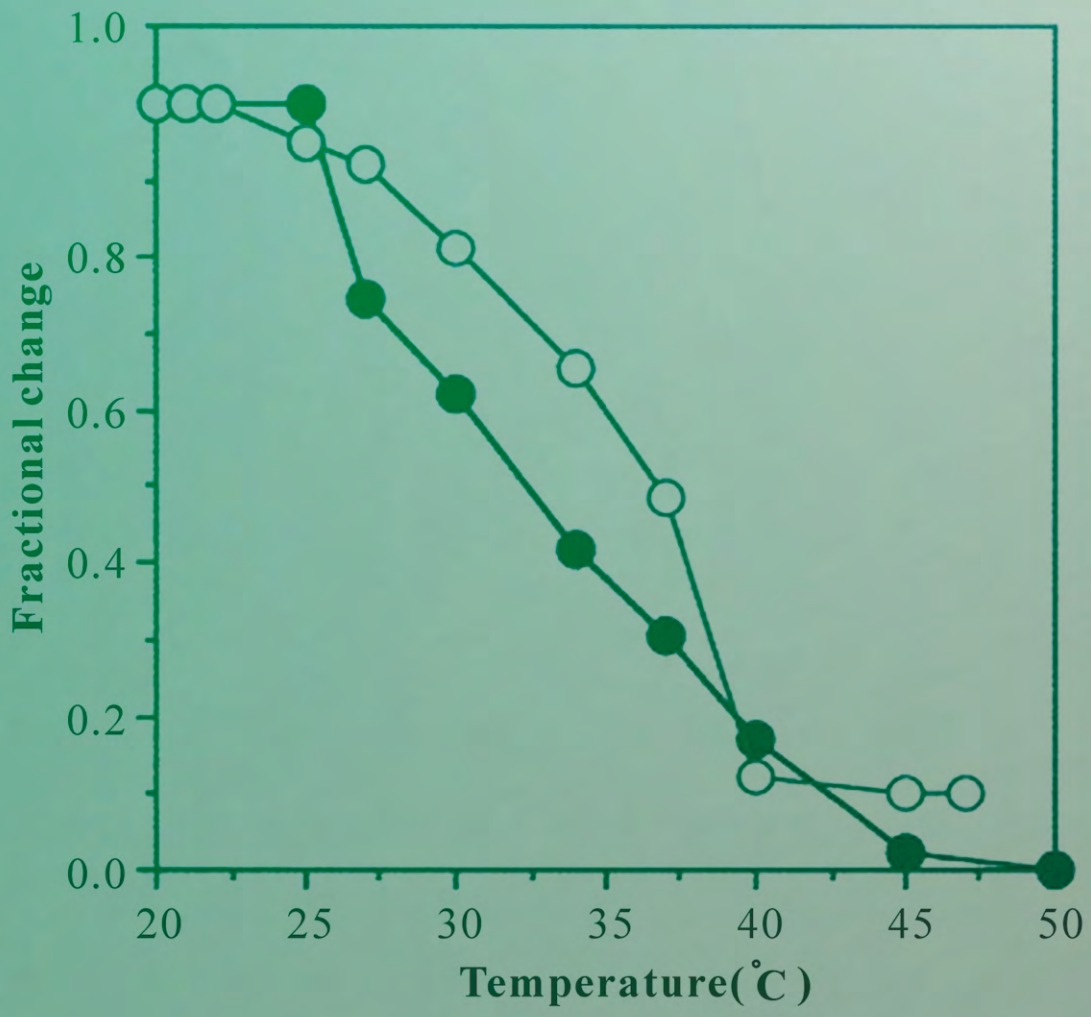

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Properties of Apricot Kernel and Oils as Fruit Juice Processing Waste

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ABSTRACT

Recently, more attention has been focused on the utilization of food processing by products and wastes, as well as under-utilization agricultural products. Some physical and chemical properties, mineral contents and fatty acid composition of apricot kernel and oils were determined. The oil yields from kernels changed from 42.2% *to* 57.2%. *The crude fibre contents ranged between* 4.06% *and* 7.63%. *In addition, crude protein contents ranged between* 15.1% *and* 24.2%. *While the peroxide values of kernel oils change between* 0.834 *meq/Kg and* 8.294 *meq/Kg, acidity values ranged between* 0.279% *and* 0.700%. *The main fatty acids in apricot kernel oils were oleic, linoleic and palmitic acids. Oleic acid contents of kernel oils varied between* 53.06% *and* 70.90%. *On the other hand, linoleic acid contents ranged between* 21.43% *and* 35,67%. *As a result, the present study showed the apricot kernels of the researched species of apricot kernels from Turkey are a potential source of valuable oil which might be used for edible and other industrial applications.*

Keywords*: *Apricot, Kernel, Oil, Fatty acid Composition, Mineral Contents

1. Introduction

Apricot (*Prunus armeniaca* L.) is a member of the Rosaceae family and is widely distributed in most countries of the world. The problems of industrial waste are becoming harder to solve, and much effort will be needed to develop the nutritional and industrial potential of by-products [1]. The kernels are considered as non-traditional potential resources for oils [2-3]. World apricot production is about 2.5 million ton. Turkey produces about 1/5 of total world apricot production. The apricot production in Turkey is 557.572 ton and 716.415 ton in 2007 and 2008 years, respectively. The one of highest apricot growing location in Turkey is Malatya province [4]. Some fruit seeds such as cherry, apricot, citrus and apple can be used as sources of oils. Some seed oils are already used for several purposes: blending with highly saturated edible oils to provide new oils with modified nutritional values as ingredients in paint and varnish formulations, surface coatings and oleo-chemicals, and as oils for cosmetic purposes [5]. Currently, large amounts of fruit seeds are discarded yearly at processing plants. This not only wastes a potentially valuable resource but also aggravates an already serious disposal problem. To be economically viable, however, both oil and meal from these fruits seeds must be utilized [6].

The aim of this study was to determine their physical and chemical properties of apricot kernel contents of several apricot cultivars collected from Malatya province in Turkey.

2. Material and Method

2.1. Material

Apricot cultivar (Hüdayi, Hacıhaliloğlu, Soğancı, Şahinbey, Hacıkız, Canino, Caona, Sakıt–2, Çekirge–52, Erkenağerik, 693-K, Karacabey, Ethembey, Alyanak, Kabaaşı, Hasanbey and Aprikoz) kernels were obtained by hand processing from apricots growing in Malatya province of Turkey in August 2008. Kernels were kept in glass jars until analyses at refrigerator. In all stages of trials, dry and mature kernels have been used.

2.2. Chemical Analyses

The some chemical compositions (crude fiber, crude oil, crude protein and crude ash) were analyses according to AOAC [7]. Viscosity values of kernel oils were measurement with Vibro (SV-10) Viscosimetry [7]. For oil analyses, each samples was homogenized and subjected to extraction for 6 h with petroleum ether (boiling range 30-60°C) in a Soxhlet apparatus. The extracted oil was

dried over anhydrous sodium sulphate and the solvent was removed under reduced pressure in a rotary film evaporator. Oil percentages were determined by weight difference. Ash was determined in a muffle furnace at 900°C for 8 h [7]. The nitrogen content estimated by the Kjeldahl method and was converted to protein content by using the conversion factor 6.25. The fiber and moisture were determined according to Demir and Özcan [8].

2.3. Determination of Fatty Acids

Fatty acid composition for apricot kernel samples were determined using a modified fatty acid methyl ester method as described by Hışıl [9]. The oil was extracted three times for 2 g air-dried seed sample by homogenization with petrolium ether. The oil samples (50-100 mg) was converted to its fatty acid methyl esters (FAME). The methyl esters esters of the fatty acids (1 µl) were analysed in a gas chromotography (Shimadzu GC-2010) equipped with a flame ionising detrctor (FID), a fused silica capillary column (60 m x 0.25 mm i.d.; film thickness 0.20 mikrometer). It was operated under the following conditions: oven temperature program. 90℃ for 7 min. Raised to 240℃ at a rate 5℃/min and than kept at 240℃ for 15 min); injector and detector temperatures, 260 and 260℃; respectively, carrier gas. nitrogen at flow rate of 1.51 ml/min; split ratio. 1/50 µl/min.

A Standard fatty acid methyl ester mixture (Sigma Chemical Co.) was used to identify sample peaks. Commercial mixtures of fatty acid methyl esters were used as reference data for the relative retention times [10]. Quantitative analyses of the fatty acids were performed using the heptadecanoic acid methyl ester as internal Standard. The results are mean values of three replicates.

2.4. Determiation of Mineral Contents

About 0.5 g of dried and apricot kernels were put into burnig cup with 15 ml of pure NHO_3. The sample was incinerated in a MARS 5 microwave oven (CEM corporation Manufactura) at 200°C. Distilled deionized water and ultrahigh-purity commercial acids were used to prepare all reagents, standards and apricot kernel samples. After digestion treatment. samples were filtrated through whatman No 42. The filtrates were collected in 50 ml Erlenmayer flasks and analysed by ICP-AES (Varian). The mineral contents of the samples were quantified against standard solutions of known concantrations which were aralyjed concurrently [11].

Working conditions of ICP-AES:

Instrument	:ICP-AES (Varian-Vista
RF Power	:0.7-1.5 kw (1.2-1.3 kw for Axial)
Plasma gas flow rate (Ar)	:10.5-15 L/min. (radial)
	15 " (axial)
Auxilary gas flow rate (Ar)	:1.5"
Viewing height	:5-12 mm
Copy and reading time	:1-5 s (max.60 s)
Copy time	:3 s (max. 100 s)

2.5. Statistical Analyses

Results of the research were analysed for statistical significance by analysis of variance [12]. This research was performed by three duplicates with a replicate.

3. Results and Discussion

The physical and chemical properties of some apricot kernels collected from Malatya province in Turkey are given in **Table 1**. The crude fibre, crude ash, crude protein, crude oil and kernel weigh values of apricot kernels were determined. According to variance analyses, important differences were found between crude cellulose, crude fibre, crude ash, crude protein, crude oil and their weighs and kinds as statistical, $p < 0,01$ level.

The oil yields of kernels varied from 42.2% (Canino cv) to 57.2% (Sakıt-2 cv) of the dry weight. The oil contents of kernels changed among the varieties to more than about 50% of each. However, because of economical value of the oil, these kernels could be used as potential sources of oils. Sakıt-2 cultivar had the highest oil content, followed by Çekirge-52 cv (54.4%), Erkeneğerik-cv (54.7%), Şahinbey cv (54.1%), Ethembey cv (53.9%), Kabaşı cv (53.9%), Hacıkız cv (53.5%) and Hacıhaliloğlu cv (52.7%). The crude fibre contents ranged between 4.06% (Sakıt-2 cv) and 7.63% (Aprikoz). While crude ash contents change between 2.138% (Kabaaşı cv) and 3.454% (Aprikoz), crude proteins of kernels ranged between 15.1% (Kabaaşı cv) to 24.2% (Caona cv). In addition, kernel weights changed between 0,300 g/unit and 0,576 g/unit (Alyanak cv), Femenia *et al*. [1] had reported that kernels of bitter and sweet apricots collected from Mallorca (Spain) contained oil between 39.7% and 47.2%; 49.8% and 56.1% oil, respectively, In other study, kernel oil contents of almond were determined between 45.9% and 61.7% [13]. Dry kernels of plum, apricot and peach contained 32%, 37% and 43% of oil, respectively [3]. Kappor *et al*. [14] obtained values of up to 67g/100 g for oil content in sweet apricot varieties. Özcan [15] determined moisture (4,91-5.12%), crude oil (45.3-51.4%), crude protein (23.58-27.70%), crude fibre (13.49-17.98%), crude ash (2.10-2.67%) and mass of 100 kernels (28.72-49.6 g) values of four apricot cultivars. These properties are effected mainly by variety [16]. However, higher protein contents (37-45 g/100g) have been reported by several authors [6,16]. The results in this work are in general agreement with these results.

Our results related to apricot (47.55 to 55.45%) was found similar values those of Kappor *et al*. [14], Femenia *et al*. [1], Özcan [15] and Matthaus and Özcan [17].

Some chemical and physical properties of kernel and oils are shown in **Table 2**. According to variance analyses, differences between varieties to peroxide value, acidity, refractive index and viscosity were found statistically important at the $p < 0,01$ level. While the peroxide values of kernel oils change between 0.834 (Soğancı) and 8.294 meq/Kg (Karacabey cv), acidity values ranged between 0,279% (Kabaaşı cv) and 0,700% (Karacabey). The highest peroxide values were established in Hasan-

Table 1. Physical and chemical properties of apricot kernel.

Cultivars	Crude fibre (%)	Crude ash (%)	Crude protein (%)	Crude oil (%)	Kernel weight (g)
Hüdayi	5.221cd	2.261ef	19.172bc	50.550defg	0.471ef
Hacıhaliloğlu	4.459fgh	2.500bcde	17.342cde	52.710bcde	0.503de
Soğancı	4.714def	2.447bcde	17.932bcde	51.975bcde	0.457fg
Şahinbey	4.159fgh	2.510bcde	20.278bc	54.110abc	0.569a
Hacıkız	4.638efg	2.808ab	18.126bcd	53.470bcd	0.552ab
Canino	6.928a	2.761bc	18.001bcde	42.195ı	0.359h
Caona	4.068gh	2.310def	24.201a	49.840efgh	0.425g
Sakıt–2	4.060h	2.577bcde	14.948bc	57.185a	0.545abc
Çekirge–52	5.099cde	2.766bc	19.265bc	54.370ab	0.554ab
Erkenağerik	4.681def	2.763bc	15.980de	54.735ab	0.521bcd
693-K	5.437bc	2.747bcd	17.403cde	47.545gh	0.566a
Karacabey	4.426fgh	2.358bcde	20.589ab	51cdef	0.552ab
Ethembey	5.371c	2.707bcd	19.215bc	53.915abc	0.511cd
Alyanak	4.195fgh	2.455bcde	17.573cde	48.300fgh	0.576a
Kabaaşı	6.027b	2.138e	15.106e	53.895abc	0.487def
Hasanbey	5.165cde	2.789bc	18.632bcd	50.370defg	0.568a
Aprikoz	7.638a	3.454a	20.879ab	47.170h	0.300ı

Table 2. Physical and chemical properties of apricot kernel oils.

Cultivars	Peroxide value (meq/Kg)	Acidity (%)	Refractive index ($n^{20}{}_{D}$)	Viscosity (Mpa)
Hüdayi	1.907bc	0.641bd	1.543bc	40.300a
Hacıhaliloğlu	1.026def	0.474ef	1.535ef	24.500h
Soğancı	0.834f	1.562a	1.538de	23.300k
Şahinbey	1.888bc	0.416f	1.530gh	26.350n
Hacıkız	0.932ef	0.696b	1.542bcd	27.350f
Canino	0.833f	0.474ef	1.546ab	34.700c
Caona	1.235def	0.447ef	1.543bc	29.350e
Sakıt-2	1.422cde	0.306ef	1.545ab	16.250n
Çekirge-52	0.986def	0.615bcd	1.533fg	23.575ık
Erkenağerik	2.210b	0.609bcd	1.543bc	14.550o
693-K	1.339def	0.528de	1.549a	13.600p
Karacabey	8.294a	0.700b	1.545ab	25.900g
Ethembey	1.199def	0.446ef	1.543b	31.150d
Alyanak	1.267def	0.584cd	1.539cd	17.550m
Kabaaşı	1.481cd	0.279ef	1.527h	35.200b
Hasanbey	7.673a	0.444ef	1.535ef	21.100l
Aprikoz	1.384cde	0.639bc	1.538de	23.85ı

bey cv (7,673 meq/Kg) and Karacabey cv (8,294 meq/Kg) samples. Refractive index was determined between 1.527 (Kabaaşı) and 1,549 (693-K cv). In addition, viscosity values of kernel oils were measured between 13,600 (693-K cv) and 40,300 (Hüdayi cv). Generally, the highest viscosity values were established in Hüdai, Canino, Kabaaşı and Ethem Bey cultivars. Differences among the values of *Prunus* varieties can probably be because of growing conditions, climatic, environmental conditions and analytic conditions.

Fatty acid composition of apricot kernel oils is given in **Table 3**. Results showed that the oils of all varieties used in this experiment had higher oleic acid content (between 53,06% and 70,90%) than those of other fatty acids. On

Table 3. Mineral contents of apricot kernels (mg/Kg).

Cultivars	Al	Ca	B	Cd	Cu
Hüdayi	22.415abc	1273.606bcd	11.729b	0.104a	2.941de
Hacıhaliloğlu	24.297abc	1238.389bcd	17.421ab	0.040a	5.987abc
Soğancı	22.239abc	1642.718abcd	12.566b	0.050a	2.642de
Şahinbey	19.510abc	2061.658abc	11.192b	0.056a	5.675abc
Hacıkız	18.003abc	1942.221abcd	11.749b	0.077a	4.306cd
Canino	19.497abc	1371.717abcd	9.743b	0.072a	1.07f
Caona	32.850a	2220.799a	57.249a	0.065a	6.496a
Sakıt–2	29.968ab	1921.818abcd	16.432ab	0.063a	6.361a
Çekirge–52	27.887ab	2238.786a	15.440b	0.057a	7.070a
Erkenağerik	17.439abc	1462.969abcd	10.522b	0.081a	1.058f
693-K	25.602abc	1792.290abcd	17.105ab	0.047a	4.474bc
Karacabey	25.480abc	2032.622abc	16.385ab	0.082a	6.214ab
Ethembey	17.697abc	1867.853abcd	11.310b	0.030a	6.222ab
Alyanak	28.882ab	2159.571ab	15.742ab	0.122a	4.283cd
Kabaaşı	9.926c	1063.019d	8.544b	0.033a	1.905ef
Hasanbey	25.779abc	1607.618abcd	11.015b	0.018a	6.004abc
Aprikoz	13.972bc	1215.081cd	10.208b	0.056a	1.317ef
Hüdayi	6090.986h	1726.441gh	187.085cd	3442.016abcd	28.529cd
Hacıhaliloğlu	8383.253f	1952.767bcde	245.196bcd	3762.980a	35.053abcd
Soğancı	8537.620ef	1931.137cdef	603.326abcd	3177.026d	28.812cd
Şahinbey	8136.141fg	1971.202bcde	705.950ab	3409.697bcd	25.051d
Hacıkız	10234.682bc	1874.072defg	862.535a	3456.812abcd	33.147bcd
Canino	9385.655cde	2040.736abcd	346.035abcd	3494.124abcd	28.954.cd
Caona	6206.455h	2028.789abcd	843.767a	3480.659abcd	42.469abc
Sakıt–2	8828.335def	1836.232efg	718.226ab	3550.150abc	53.004a
Çekirge–52	9712.421bcd	2115.933a	699.388abc	3514.461abcd	30.923bcd
Erkenağerik	9706.068bcd	1607.314hı	585.373abcd	3209.966cd	32.794bcd
693-K	8927.422def	2043.620abc	426.582abcd	3689.357ab	32.695bcd
Karacabey	7308.140g	1929.373cdef	782.416a	3583.744ab	34.604bcd
Ethembey	8389.089f	2114.926ab	452.838abcd	3471.977abcd	33.212bcd
Alyanak	8630.503ef	1995.662abcde	552.593abcd	3448.382abcd	45.766ab
Kabaaşı	8614.027ef	1959.762bcde	155.921d	3613.276ab	42.467abc
Hasanbey	10334.982b	1778.782fg	777.275a	3547.449abc	33.483bcd
Aprikoz	12715.908a	1513.934ı	503.524abcd	3399.972bcd	22.489d

the other hand, linoleic acid contents of kernel oils ranged between 21.43% (Sakıt-2 cv) and 35.67% (Aprikoz cv), As an be observed, the proportions of the most abundant fatty acids (oleic acid) of the kernel oils varied among different varieties. This proportion was also higher than that in other fruit seed oils; mahaleb (35,4%), cherry laurel (53,7%), peanut (56,27%), date pit (49,54%), walnut (13,8-33,0%) [18-21]. Palmitic acid is the main saturated component in all apricot cultivars. Palmitic acid is differed in the different apricot cultivars. Its percentage was found between 4,56% (Caona cv) and 6,03% (Aprikoz cv). The high concentration of linoleic acid in apricot kernel oils make these oils have high nutritional value as linoleic acid is one of the three essential fatty acids [11]. These results are in good agreement with in fatty acid composition for several *Prunus* kernels [1,3,15,17,20,22]. Matthaus and Özcan [17] determined from 43.9% to 78.5% oleic, from 9.7% to 37.0% linoleic and from 4.9% to 7.3% palmitic acids in some *Prunus* cultivars.

The ability of some unsaturated vegetable oils to reduce the serum cholesterol level may focus attention on the apricot kernel oil due to its high unsaturated oil content [1]. Our results are similar in fatty acid composition when compared to the values in the literature.

The mineral contents of apple kernels were determined by ICP-AES. The mineral compositions of kernels are summarized in **Table 4**. Mineral elements were found to vary widely depending on different apricot cultivar kernels. According to variance analyses, differences between apricot cultivars to Cu, K and Mg were found statistically important at $p < 0,01$ level. Also, differences of cultivars had important at the $p < 0,05$ on Zn element.

Ca, K, Mg, Na and P contents of all the apricot cultivar kernels were generally found very high. In addition, other minerals were determined very low. The levels of Ca of samples ranged between 1063.0 mg/Kg (Kabaaşı cv) and 2238.8 mg/Kg (Çekirge-52 cv), K contents were determined between 6206.5 mg/Kg (Caona cv) and 12715.9 mg/Kg (Aprikoz). While Mg contents are established between 1513.9 mg/Kg (Aprikoz cv) and 2115.9 mg/Kg (Çekirge-52 cv), P contents of kernels were found between 3177.0 mg/Kg (Soğancı cv) and 3762.9 mg/Kg (Hacıhaliloğlu cv). According to report of Femenia *et al.* [1], phosphorus, magnesium and calcium amounts ranged between 1100-1200 mg/100 gin apricot kernels. Also, the other essential elements; iron, manganese, copper, and zinc, account for 7-9,5 mg/100 g [1]. Average mineral contents of apricot varieties were found to be between 2.73-3.68% for Na, 1.06-2.94 ppm for P, 0.35-0.64 ppm for K, 0.08-0.11 ppm for Ca, 0.23-0.26 ppm for Mg, 10.7-74.9 ppm for Fe, 11.80-42.35 ppm for Zn, 0.73-1.46 ppm for Mn and 1.10-4.76 ppm for Cu [15].

These differencies of cultivars minerals may be due to growth conditions, varieties, genetic factors, harvesting time, soil properties, geographical variations and analytical procedures [23,24]. Calcium is the major component of bone and assists in teeth development [25]. Other elements

Table 4. Fatty acid composition of apricot kernel oils (%).

Cultivars	K	Mg	Na	P	Zn
Hüdayi	6090.986h	1726.441gh	187.085cd	3442.016abcd	28.529cd
Hacıhaliloğlu	8383.253f	1952.767bcde	245.196bcd	3762.980a	35.053abcd
Soğancı	8537.620ef	1931.137cdef	603.326abcd	3177.026d	28.812cd
Şahinbey	8136.141fg	1971.202bcde	705.950ab	3409.697bcd	25.051d
Hacıkız	10234.682bc	1874.072defg	862.535a	3456.812abcd	33.147bcd
Canino	9385.655cde	2040.736abcd	346.035abcd	3494.124abcd	28.954.cd
Caona	6206.455h	2028.789abcd	843.767a	3480.659abcd	42.469abc
Sakıt–2	8828.335def	1836.232efg	718.226ab	3550.150abc	53.004a
Çekirge–52	9712.421bcd	2115.933a	699.388abc	3514.461abcd	30.923bcd
Erkenağerik	9706.068bcd	1607.314hı	585.373abcd	3209.966cd	32.794bcd
693-K	8927.422def	2043.620abc	426.582abcd	3689.357ab	32.695bcd
Karacabey	7308.140g	1929.373cdef	782.416a	3583.744ab	34.604bcd
Ethembey	8389.089f	2114.926ab	452.838abcd	3471.977abcd	33.212bcd
Alyanak	8630.503ef	1995.662abcde	552.593abcd	3448.382abcd	45.766ab
Kabaaşı	8614.027ef	1959.762bcde	155.921d	3613.276ab	42.467abc
Hasanbey	10334.982b	1778.782fg	777.275a	3547.449abc	33.483bcd
Aprikoz	12715.908a	1513.934ı	503.524abcd	3399.972bcd	22.489d

which may contribute to biological processes, but which have not been established as essential, are barium, cadmium [23]. The high quantity of potassium, phosphorus, magnesium, and calcium, together with the small proportion of sodium plus the content of the essential elements as iron, manganese, copper, and zinc and allows the apricot, as well as the almond, to be considered as an excellent source of bioelements [26].

4. Conclusions

The accurate quantification of these analyses has very important applications for the nutrition sciences, because fatty acids, protein, oil and mineral contents in particular seed have a very important effect on health. These results of the experiment presented have shown that apricot cultivars have some distinctive chemical and physical properties, fatty acid and mineral content profiles. Kernels in apricot varieties can be a good source oil due to their abundance in the kernels and their high oil content. Such utilization of apricot fruits processing wastes could provide extra income and at the same time help minimize a waste disposal problem. The mineral contents of apricot cultivar kernels collected from Malatya province of Turkey were established by ICP-AES. The contents of most minerals such as Ca, K, Mg and P are at adequate levels. Mineral elements were found to vary widely depending on different apricot kernels. Apricot kernels were found to be important sources of nutrients and essential elements. In addition, it is apparent that apricot kernels are good sources of micro-and macro minerals, and consumed as a food ingredient to provide the human nutrient.

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Effect of Virgin Coconut Meal (VCM) on the Textural, Thermal and Physico Chemical Properties of Biscuits

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ABSTRACT

Biscuits were prepared by incorporating virgin coconut meal (*VCM*) *into refined wheat flour* (*maida*) *at* 5-25% *level and evaluated for physical, chemical, nutritional, textural and sensory attributes. All the prepared biscuits had high protein and fiber contents as compared to control* (100% *refined wheat flour based product*). *With the incorporation of VCM, hardness and toughness of dough increased while there was a decrease in stickiness and adhesiveness values. Incorporation of VCM had a significant effect on color values of biscuits as the concentration of VCM was increased. The values of L* decreased while those for a* and b* increased. Sensory analysis revealed that* 15% *VCM biscuits were the most acceptable. The Differential scanning calorimetry* (*DSC*) *analysis revealed that the onset* (*To*) *decreased while end set* (*Tc*) *and enthalpy of gelatinization* (*ΔH*) *increased with the increase level of VCM.*

Keywords*: *Virgin Coconut Meal* (*VCM*), *Biscuits, Dough, Texture Profile, Thermal Profile, Sensory Evaluation

1. Introduction

The term biscuit is used in Britain to describe a flat crisp baked product. Biscuits are chemically leavened, ready to eat, quick snacks with good eating quality and long shelf life [1]. The basic constituent of biscuit is flour, water, sugar and fat. The variation in these constituents causes the changes in textural properties of biscuits [2]. Biscuits are highly popular among the large segment of population in urban and rural places and its demand and consumption are increasing by leaps and bounds. Children also like the biscuits as these are available in different attractive shape and size as well as taste and palatability. The bakery industry are one of the largest organized food industries all over the world and particularly biscuits are one of the most popular products because it is economically cheaper as well as consider to be luxurious gifts for infants and school going children who are under weight [3]. Bakery products are generally used as a source for incorporation of different nutritionally rich ingredients for their diversification [4].

Various types of nutritious biscuits have been prepared by fortifying the wheat flour with various types of oil seed meals like soy flour [5], peanut [6], corn germ flour [7], cotton seed flour [8], sunflower kernel [9], safflower protein isolate [10], and coconut residue [11], and received popularity being nutritionally rich in protein and vitamins.

Nowadays, emerging beneficial oil that comes in picture of world is Virgin coconut oil [12]. The major difference between coconut oil and virgin coconut oil is the method of extraction. The general common method for the preparation of coconut oil is drying and later extraction of oil from kernal in general called “copra”. On the other hand, virgin coconut oil is extracted from fresh coconut (not copra) meat by mechanical or natural means under controlled temperature. Usually, meal obtained after the extraction of oil, have been found in animal use feed like poultry, fish and swine industry. However, meal obtained after oil extraction still possess good nutritional properties which could be utilized for value addition of various processed foods. Many in vitro studies have been performed on the animal to check the physiological effect like egg production performance in single comb white leghorn layers [13], average daily weight gain, average daily feed intake and feed conversion ratio on pigs [14], nutrient digestibility in hens [15], egg component and yolk fatty acid composition [16], growth rate of broiler chick [17] after supplementation of coconut oil

cakes. Dairo [18] has reported the protein quality indices of sun dried coconut oil meal, oven dried coconut oil meal and fermented coconut oil meal on rats. However, data on the use of VCM in the development of various processed foods are scanty. Hence, the objective of present investigation were to develop nutritious virgin coconut meal based biscuits and study the changes in the physico-chemical, textural and thermal characteristics.

2. Materials and Methods

2.1. Raw Materials

Refined wheat flour was procured from local market. Virgin coconut meal was supplied by Central Plantation Crops Research Institute (CPCRI), kasargod, Kerala, India after the extraction of VCO. The VCM was powdered in an ultra centrifugal mill (Retsch RI, Germany) using 0.5 mm sieve.

2.2. Preparation of Biscuits

Biscuits were prepared by the incorporation of VCM with the replacement of refined wheat flour at the level of 5, 10, 15, 20 and 25% in the standardized formulations. The preliminary step for the preparation of biscuit was creaming. The creaming was carried out by using shortening i.e. hydrogenated fat and sugar in Hobart Mixer for 4 min and subsequently added a mixture of refined wheat flour and baking powder along with measured quantity of water in the Hobart mixer. The mixing was done for five minutes till the soft dough was formed. The prepared dough was subjected to sheeting of 4 mm thickness manually. Finally sheets were cut to 4.5 cm diameter by using the die and were subjected to baking at 150°C for 30 min.

2.3. Nutritional Analysis

Moisture, protein, fat, ash and crude fiber were determined as per AOAC method [19]. Fatty acid profile of extracted fat from biscuit samples were determined as AOCS [19] method by using gas liquid chromatograph (Model Chemito 8510 HR, Mumbai, India) with 10% diethylene glycol succinate column (DEGS 8“X1/8”). The minerals like Ca, Fe, Zn, Na, K were determined by Atomic Absorption Spectrometer (AAS Varion 6, Analytik Jena AG, Germany) at 422.7, 248.3, 213.9, 589.0 and 766.5 nm wavelength respectively. For determining minerals the biscuits were converted into ash in muffle furnance. After ashing, 15 ml of 3 N HCl was added to the crucible having ash material and boiled until it was reduced to 2-3 ml. The volume was made up to 50 ml in volumetric flask and mineral content estimated by AAS by flame mode and results were expressed in mg/100 g of sample.

2.4. Physical Property

Biscuits were cooled for 1hrs for the determination of spread ratio as per the method described in AACC [20], six biscuits were taken randomly and placed them edge to edge and stacking for the estimation of diameter and thickness and finally by the subtraction of their average value spread ratios were obtained.

2.5. Texture Properties of VCM Based Biscuits

Texture attribute of biscuits like hardness, cutting force, breaking strength, toughness properties were taken as quality parameters to check the textural properties of VCM based dough and biscuits by using TAHDI Texture analyzer (Stable Micro System Ltd. London, UK) using a 25 Kg load cell and the application programme provided with the apparatus (Texture Expert for Windows, TM version 1.22). Hardness and cutting strength of biscuits were determined as mentioned by Singh *et al.* [1]. Both were measured using cylindrical probe P/2 and HDS/BS blade respectively with 25 Kg of load cell. For hardness some minor modifications in TA settings were performed, *i.e.*, test speed kept at 2 mm/s. The first peak of graph was considered as initial fracture force and absolute peak force was considered as the hardness of the biscuit.

2.6. Texture Properties of VCM Based Dough

Hardness and toughness of dough was determined as method mentioned in Tyagi *et al.* [21] with some minor modifications using 40 mm cylindrical cup, probe 10mm and settings were kept as pre test speed of 2 mm/s, test speed of 3 mm/s, post test speed of 10 mm/s with 25 Kg load cell and strain was set at 60%. The obtained peak force was considered as Hardness of dough and area under curve was considered as toughness of dough. Dough stickiness fixture having 25 mm diameter has been used to measure the stickiness and adhesiveness of dough. The pre test speed, post test speed and test speed were same *i.e.*, 10 mm/min and clearance, hold time, compression force were 3 mm, 10 s and 10 N respectively.

2.7. Color Measurements

Changes in the crust color of biscuit by the incorporation of VCM were measured by CIE color values [L*(lightness), a*(redness), b*(yellowness)] using Hunter colorimeter D-65 illuminant and 10° observer. The equipment (Mini Scan XE Plus, Model 45/0-S, Hunter Associates Laboratory Inc, Reston, VA, USA) was calibrated using white and black standard ceramic tiles and the readings were recorded with inbuilt software.

2.8. Thermal Properties

The thermal properties of biscuit were determined by using DSC-821 (Mettler Toledo, Switzerland) equipped with a thermal analysis data station. The samples were weighed into a 40 µl capacity aluminum pan (Mettler, ME 27331) and distilled water was added with the help of Hamilton micro-syringe. The samples were hermetically sealed and allowed to stand for 30 min at room temperature before heating in DSC. The DSC analyzer was calibrated using indium and an empty aluminum pan was used as reference sample pans. Both were heated at the rate of 10°C/min from 20°C to 200°C for the estimation endothermal changes as the temperature increased.

2.9. Sensory Properties

Sensory quality of VCM biscuits were evaluated by a panel of 15 semi trained judges by grading for color, taste, texture and overall acceptability on 9 point hedonic scale, with 9 as excellent in all respects and 1 for unacceptable samples [21].

2.10. Statistical Analysis

The data analysis, for Duncan multiple comparisons and response optimization were done using STATISTICA stat software release 8.0 package.

3. Results and Discussion

3.1. Proximate Composition of VCM

Virgin coconut meal contains 10.8 ± 0.62% moisture, 54.98 ± 1.05% fat, 14.9 ± 0.25% protein, 15.2 ± 0.2% crude fiber, 2.54 ± 0.05% ash content and 1.57 ± 0.02% carbohydrate (by difference). It is a rich source of minerals like calcium (83.78 ± 0.015 mg/100 g), Sodium (98.77 ± 0.03 mg/100 g) and potassium (1700 ± 0.15 mg/100 g). Iron and zinc are present comparatively lower concentration, *i.e.*, 22.76 ± 0.14 and 2.57±0.05 mg/100 g respectively. Its water absorption capacity and solubility index were 4.82 g/g and 0.45 g/g respectively. Major fatty acid present in VCM was lauric acid (54.37%) followed by myristic acid (20.13%), palmitic acid (8.61%), capric acid (5.81%), oleic acid (5.49%), stearic acid (3.14%), linoleic acid (1.55%) and caprylic acid (0.69%).

3.2. Proximate Composition and Nutritional Value of VCM Biscuits

Proximate composition of biscuits prepared by using VCM (5-25%) is given in **Table 1**. It is evident that incorporation of VCM resulted change in moisture content from 3.32 to 4.46%, fat 24.79 to 39.34%, protein 4.56 to 5.97%, ash content 0.26 to 0.73% and fibre 0.15 to 3.55%. The statistical analysis of proximate composition revealed that there was no significant difference in moisture content up to 15% incorporation of VCM but addition of VCM above 15% level, showed significant increase in moisture content ($p > 0.05$). This may be due to high water absorption capacity of meal, *i.e.*, 4.82 g/g which gradually increase with rise in the concentration of VCM in biscuits. Similar, results has been reported by Talati *et al.* [23] and Tyagi *et al.* [22] in mustard flour biscuit. The incorporation of VCM (5-25%) resulted in considerable increase in the fat, protein, fibre and ash content of biscuit as mentioned in **Table 1**. Control biscuits samples contained oleic acid as the major fatty acid followed by palmitic, stearic acid, myristic acid, lauric acid, capric and caprylic acid. Addition of 5-25% VCM in biscuits recipe resulted significant decreased in oleic acid and palmitic acid while myristic acid, lauric acid, capric and caprylic increased as figured in **Table 2**. Lauric acid is a medium chain fatty acid, which has the additional beneficial function of being formed into monolaurin in the human or animal body. The mineral content of biscuits was tabulated in **Table 1**. As the VCM content increased from 5-25% in refined wheat flour, the mineral content, particularly calcium, potassium, iron and sodium contents were increased and improved the nutritional quality characteristic of biscuits. Calcium content was increased from 9.55 to 24.35 mg/100 g, potassium 59.24 to 442.06 mg/100 g, iron 1.05 to 5.97 mg/100 g, zinc 0.22 to 0.73 mg/100 g and sodium 99.37 to 132.35 mg/100 g (**Table 1**).

3.3. Effect on Physical Properties of VCM Biscuits

Spread ratio was slightly increased with rise in concentration of VCM in biscuits (**Table 3**). This may be due to the increase in fat content in biscuits with higher VCM. The similar results were reported with corn and potato flour biscuit [24], Oat bran biscuit [4] and raw, toasted wheat bran biscuit [25]. Some authors reported that spread factor is highly affected by fat content, as the content of fat increased spread ratio gradually increased [26] or some had been supported this effect due to the increased extension of the dough [27].

3.4. Influence of VCM on Textural Properties of Biscuits

Breaking strength is one of the criteria to measure the biscuit hardness [21] and results for this parameter were compiled in **Table 3**. The results indicated that breaking strength of the VCM biscuits containing 5% VCM had no significant difference with control but the treatments involving with sample more than 5% VCM resulted significantly ($p > 0.05$) decrease in breaking strength. Earlier also similar trends were observed by addition of cow pea flour incorporated biscuits [28]. The average maximum cutting strength (**Table 3**) values significantly decreased from control sample with incorporation of VCM. In the

Table 1. Chemical and nutritional components of biscuits containing VCM (n = 3).

VCM (%)	Moisture (%)	Fat (%)	Protein (%)	Ash content (%)	Fiber (%)	Ca (mg/100 g)	Zn (mg/ 100 g)	Na (mg/ 100 g)	K (mg/100 g)	Fe (mg/100 g)	Ca (mg/100 g)
0	3.32a	24.79a	4.56a	0.26a	0.15a	9.55a	0.22a	99.37a	59.24a	1.05a	9.55a
5	3.38a	28.62b	4.78b	0.34b	0.86b	10.51a	0.30ab	104.21a	127.22b	1.78b	10.51a
10	3.48a	31.08c	5.25c	0.44c	1.55c	15.99b	0.39b	120.48b	151.33c	3.03c	15.99b
15	3.56a	32.39d	5.52d	0.53d	2.26d	18.26c	0.52c	123.47b	282.38d	3.65c	18.26c
20	4.04b	36.81e	5.82d	0.62e	2.9e	22.47d	0.58d	130.55c	365.14e	5.07d	22.47d
25	4.46c	39.34f	5.97e	0.73f	3.55f	24.35d	0.73d	132.35c	442.06f	5.97e	24.35d

Mean values with the same superscript letters within the same column do not differ significantly ($p > 0.05$)

Table 2. Fatty Acid Profile (%) of VCM Biscuits (n = 3).

VCM (%)	Capric (%)	Caprylic (%)	Lauric (%)	Myristic (%)	Palmitic (%)	Stearic (%)	Oleic (%)
0	0.75 ± 0.07*	0.45 ± 0.04	0.94 ± 0.04	2.11 ± 0.02	42.95 ± 0.02	4.99 ± 0.01	47.77 ± 0.03
5	0.86 ± 0.12	0.61 ± 0.05	5.98 ± 0.01	3.25 ± 0.08	39.72 ± 0.03	4.48 ± 0.02	45.05 ± 0.01
10	1.14 ± 0.05	1.54 ± 0.01	9.98 ± 0.01	5.29 ± 0.08	38.14 ± 0.05	4.49 ± 0.07	39.40 ± 0.06
15	1.17 ± 0.01	1.64 ± 0.04	11.39 ± 0.01	6.26 ± 0.14	36.56 ± 0.03	4.44 ± 0.02	38.53 ± 0.07
20	2.17 ± 0.01	1.77 ± 0.02	14.47 ± 0.10	6.93 ± 0.01	32.29 ± 0.06	4.36 ± 0.03	37.54 ± 0.02
25	2.27 ± 0.02	1.82 ± 0.01	17.39 ± 0.06	8.56 ± 0.08	29.34 ± 0.04	4.18 ± 0.03	34.77 ± 0.07

*Mean ± S. D

Table 3. Textural Properties of biscuits containing VCM (n = 3).

VCM (%)	Spread ratio	Breaking strength (N)	Hardness (N)	Cutting force (N)
0	9.63^{a}	68.89^{a}	10.90^{a}	46.23^{a}
5	9.67^{a}	69.09^{a}	9.98^{a}	39.56^{b}
10	9.72^{ab}	63.32^{b}	9.38^{a}	37.59^{bc}
15	9.81^{b}	47.77^{c}	9.49^{a}	35.44^{cd}
20	9.92^{c}	21.84^{d}	7.21^{b}	33.21^{d}
25	9.88^{c}	16.54^{e}	6.22^{b}	28.09^{e}

Mean values with the same superscript letters within the same column do not differ significantly ($p > 0.05$)

Table 4. Effect of incorporation of VCM on textural characteristics of biscuit dough (n = 3).

VCM (%)	Hardness of dough (N)	Toughness (Nmm)	Stickiness (N)	Adhesiveness (Nmm)
0	6.14^{a}	26.42^{a}	3.58^{a}	1.56^{a}
5	6.44^{a}	26.62^{a}	3.35^{a}	0.89^{b}
10	6.69^{a}	42.14^{b}	3.07^{ab}	0.71^{bc}
15	7.69^{b}	42.62^{b}	2.58^{bc}	0.66^{c}
20	8.09^{b}	43.69^{b}	2.52^{bc}	0.67^{c}
25	8.55^{b}	46.05^{c}	2.19^{c}	0.57^{c}

Mean values with the same superscript letters within the same column do not differ significantly ($p > 0.05$)

case of hardness there was no significant difference showed till the level of 15% and then there was significant decrease showed at 20-25% incorporation level.

3.5. Influence of VCM on Textural Properties of Dough

Hardness and toughness of dough showing increment in values from 6.14 N to 8.55 N and 26.42 Nmm to 46.05 Nmm respectively. Dough hardness value indicated a significant difference especially caused by the 15 to 25% incorporation of VCM from control. As VCM is rich in fiber therefore, percentage increment in VCM leads to harder dough as earlier also some researcher reported similar trend with wheat and rice bran incorporated biscuit dough [4]. Stickiness of dough is important for sheeting of biscuits. Substitution of VCM in different percentage was associated with a decrease in dough stickiness. There was no significant difference in dough stickiness between VCM containing dough and control up to 10% replacement of VCM. Further increment in VCM significantly ($p > 0.05$) decreased the stickiness. The results indicated that control dough was much stickier than the VCM containing (5-25%) dough with different levels. Decrease in

stickiness of dough due to presence of VCM may be attributed to the larger particle size of VCM (0.5 mm) than the refined flour (0.15 mm) as the larger particle absorbed more water and free water content for dough formation become less and resulted decrease in stickiness [29].

3.6. Influence of VCM on Color Values of Biscuits

Surface browning is a common phenomenon for biscuits during baking. Brown pigments are formed in the advanced stages of browning reactions and can be measured by color determination. Color measurements provide a useful index to evaluate the intensity of browning reactions and have been used to monitor the processing of bread [30]. In the present study the L* value of VCM biscuit decreased while a* and b* value increased with increased proportion of VCM (**Table 5**). The increase in a* and b* stimulus may be due to increase in redness and yellowness during baking. The similar trend has been reported biscuits fortified with corn and potato flour [24]. The decreased L*values and increased a* and b* values resulted in darkening of the biscuits, which ultimately affected the sensory scores for colour.

3.7. Effect on Thermal Properties of VCM Biscuits

The thermal properties of the biscuits were examined using Differential scanning calorimetry and results were shown in **Table 6**. The onset temperature (T_o) significantly decreased while end set temperature (T_c) and enthalpy of gelatonization (ΔH) significantly ($p > 0.05$) increased from 60.78 to 59.04°C, 86.74 to 98.75°C and 14.87 to 90.53 J/g respectively for 5 to 25% incorporation of VCM. In present study Tc and ΔH were higher than control because of the presence of other ingredients in the system, especially VCM. As percentage of fiber increased with increasing level of VCM it may be competing with starch for water absorption and hence limiting starch swelling and gelation events resulting in higher T_c value similar findings has been reported in pasta containing pea fiber [31]. As per result obtained in this study suggest that the enthalpy of a complex increased with increasing VCM concentration. It may be due to increase in moisture content as earlier researchers have reported that the gelanization enthalpy increased with increasing moisture content [32,33].

3.8. Changes in Sensory Quality

Results of sensory evaluation in terms of sensory attributes such as color, taste, texture and Overall acceptability showed that VCM incorporated biscuits were in acceptable range (**Table 7**). Substitution of refined wheat flour with VCM up to 15% did not change the sensory attributes of biscuits. However, further addition of VCM (above 15%) significantly decreased all the sensory parameters of the biscuits. Based on the results, biscuits contained up to 15% VCM were rated as the most acceptable as their overall acceptability was comparable to the control samples (without VCM).

Table 5. Effect of VCM on color values of biscuit crust (n = 3).

VCM (%)	L*	a*	b*
0	55.06^{a}	5.55^{a}	21.41^{a}
5	54.85^{a}	6.03^{a}	21.69^{a}
10	54.91^{a}	6.16^{ab}	22.49^{a}
15	54.31^{b}	7.57^{bc}	22.68^{a}
20	52.15^{b}	7.73^{c}	25.10^{b}
25	50.94^{b}	8.91^{c}	25.15^{b}

Mean values with the same superscript letters within the same column do not differ significantly ($p > 0.05$)

Table 6. Effect of VCM on the onset, endset temperature and enthalpy of gelatinization of biscuits (n = 3).

VCM (%)	T_o(°C)	T_c(°C)	ΔH(J/g)
0	60.78^{a}	86.74^{a}	14.87^{a}
5	59.64^{b}	88.79^{b}	45.93^{b}
10	59.44^{b}	89.08^{b}	61.87^{c}
15	59.43^{b}	89.89^{b}	74.77^{d}
20	59.25^{b}	93.81^{c}	84.66^{e}
25	59.04^{b}	98.75^{d}	90.53^{f}

Mean values with the same superscript letters within the same column do not differ significantly ($p > 0.05$)

Table 7. Sensory parameters of biscuits containing VCM (n = 3).

VCM (%)	Color	Taste	Texture	Overall acceptability
0	8.30^{a}	8.17^{a}	8.16^{a}	8.20^{a}
5	8.00^{a}	8.10^{a}	8.07^{a}	8.20^{a}
10	8.10^{a}	8.13^{a}	8.13^{a}	8.20^{a}
15	8.00^{a}	8.01^{a}	8.00^{a}	8.13^{a}
20	7.37^{b}	6.97^{b}	7.06^{b}	7.20^{b}
25	6.87^{c}	6.80^{b}	6.80^{b}	6.47^{c}

Mean values with the same superscript letters within the same column do not differ significantly ($p > 0.05$)

4. Conclusions

From the above investigation it can be concluded that though biscuits containing 20-25% VCM were nutritionally rich but scored lower sensory attributes than the ones prepared from 15% VCM. Textural property of dough clearly showed that hardness and toughness were increased while stickiness and adhesiveness decreased. Color characteristics of biscuits such as L*, a*, b* values

varied significantly with addition of VCM than the control. The data obtained from thermal property showed that onset (T_o), endset (T_c) temperatures and enthalpy of gelatinization (ΔH) got affected by VCM. The study established that the incorporation of 15% VCM results in more nutritious product with acceptable sensory and textural characteristics.

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Scientific Research

Optimization of Bioactive Fortification in Apple Snacks through Vacuum Impregnation Using Response Surface Methodology

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ABSTRACT

Fortification of apple slices with t-resveratrol glucoside was obtained by vacuum impregnation (*VI*) *of apple slices with a solution containing grape juice using response surface methodology* (*RSM*). *Three independent variables of the VI process*: *vacuum pressure, application time and relaxation time, were coded at five levels in a central composite design. The VI-treated and dried apple slices were analyzed for t-resveratrol glucoside concentration and textural attributes* (*gradient and linear distance*). *Multiple response optimization revealed the following optimum VI conditions for the bioactive enrichment and improved textural quality of dried apple slices: vacuum pressure of* 6 *in. of Hg, application time of* 10 *min, and relaxation time of* 22.5 *min.*

Keywords*: *Apple Snack, Bioactive Fortification, Response Surface Methodology, Texture, Vacuum Impregnation, Resveratrol

1. Introduction

The leading causes of premature deaths in North America are cardiovascular diseases (CVD) and cancer. Scientific evidence reveals that apple, which is rich in many health-enhancing phytonutrients including dietary fiber, flavonoids and phenolic acids, provides demonstrated health benefits beyond basic nutrition and can reduce the risk of certain chronic diseases [1,2]. Considering the health benefits of apples and their suitability for snack production, apples can be used as a source for the production of healthy snacks as well as a food matrix to deliver essential nutrients and health promoting bioactives not present in apples. Among the bioactive compounds, resveratrol has gained much attention because of its chemopreventative properties [3]. Resveratrol acts as both a free radical scavenger and a potent antioxidant due to its promotion of the activities of a variety of antioxidant enzymes [3,4]. Application of vacuum impregnation (VI) process using fruit juices and sugar syrups containing minerals, vitamins, bioactives and other food ingredients is an excellent way of producing fortified or value-added fruit products [5,6]. VI can also be used to modify the compositional, thermal and physico-chemical properties of food products which will increase the process efficiency in a manner beneficial for attaining desired product characteristics such as improved taste, texture, and shelf life [7,8].

The specific objectives of the present research were to optimize the VI process conditions for the fortification of the apple slices with natural bioactive (*t*-resveratrol glucoside) present in grape juice, and to evaluate simultaneously the effect of VI on the textural quality attributes of the VI-treated and dried apple slices.

2. Materials and Methods

2.1. Plant Material and Chemical Reagents

'Empire' apple were obtained from a local fruit market (Sterling Fruit Market, Truro, NS). A solution of Welch's grape cocktail frozen concentrate diluted to a concentration of 15 ± 2° Brix was used for dipping the apple slices into solution. *t*-Resveratrol glucoside standards were obtained from ChromaDex Inc., Irvine, CA. Acetone, acetonitrile, formic acid and methanol were purchased from Fisher Scientific Ltd., ON.

2.2. Sample Preparation

Apples were cut into 2.0-mm-thick slices perpendicular to the core using an apple slicer (Waring PRO^{TM}, Model: FS 150C, Torrington, CT). The apple slices were then immediately immersed in diluted grape fruit juice (15 ± 2° Brix) with a fruit to solution ratio of 1:10 (w/v) and given VI treatment (described in Subsection 2.3) at room temperature (20 ± 2°C) using vacuum oven (Shel lab, Model: 1465, Geneq Inc., Montreal). The time to reach intended vacuum was 30 s and included in the application time. Three replicates were used for each treatment where a replicate consisted of six randomly selected slices prepared from two apples. After the VI treatment, the slices were immediately put in the vacuum dryer and drying was carried out (30-40 ± 2°C; 20 hr). Preliminary experiments were conducted to optimize the drying conditions. Immediately after drying, the vacuum impregnated dried apple slices were transferred to air tight plastic containers and kept at room temperature.

2.3. Selection of the Parameters of VI Process

For the present study, the values were selected based on the range obtained from the reported levels given in the literature review [8-14]. Coding was used as a part of the response surface methodology (RSM) and coded values are given in brackets: application time 5 (−1) to 15 (+1) min, relaxation time 15 (−1) to 30 (+1) min and vacuum pressure 4 (−1) to 8 (+1) in. of Hg, as shown in **Table 1**. Grape juice was selected as the immersion solution to act as a visual indicator of the incorporation of the grape juice by providing purple color to the apple slices and act as a source for the incorporation of the bioactive phenolic compounds, *i.e.*, *t*–resveratrol glucoside [15] which is not present in the apples and hence can be used as a marker to assess the impact of VI process conditions.

2.4. Optimization of VI Process Using RSM

To determine the optimal level for the given factors, RSM was used [16]. RSM enables the evaluation of the effects of several process variables and their interactions on response variables. This methodology has been applied by several other researchers for the optimization of food processing conditions to obtain desired quality of food products [13,17,18]. The experimental design employed was a central composite design with 3 variables and 5 levels of each variable. The three independent variables for the vacuum impregnation process were vacuum pressure, application time and relaxation time **Table 1**. The responses included fortification effect of grape juice on *t*-resveratrol glucoside concentration and textural attributes (gradient and linear distance) of the VI-treated and dried apple slices. RSREG procedure of SAS Institute, Inc. (2003) [19] was used to obtain predictive models. Optimization of the independent variables was conducted by employing canonical analysis [16]. The assumptions of normality and constant variance were checked and confirmed. Adequacy of the models was determined by analysis of variance. When the results showed a saddle point in response surfaces, the ridge analysis of SAS RSREG procedure was used to compute the estimated ridge of the optimum response at points of increasing radii from the center of the design. Contour plots were generated as a function of two factors when the third factor was held constant from the models using MINITAB15.

2.5. Determination of T-Resveratrol Glucoside Concentration

For the determination of *t*-resveratrol glucoside concentration, a previously reported method [20] was modified and used. VI-treated and dried apple slices were ground in to powder form (1 mm mesh; Cuisinart, Model: DCG-12BCC, Cuisinart Canada, Woodbridge, ON). Extraction solvent (15 mL; 40: 40: 20: 0.1 methanol: acetone: water: formic acid) was added to 0.5 g of powder and the mixtures were subjected to approximately 20 kHz frequency of sonication (Model: 750D, ETL Testing Laboratories Inc., Cortland, NY) for 15 min × three times with 10-min intervals. The crude extract was centrifuged (Model: Durafuge 300, Precision, Winchester, VA) at 4000 g for 15 min. The extracted samples were concentrated 10-fold by removal of methanol using a vacuum

Table 1. The VI process variables and their levels in central composite design.

	Levels of operating parameters				
Coded value	−1.68	−1	0	+1	+1.68
Uncoded variables					
VP	2.6	4	6	8	9.4
AT	1.6	5	10	15	18.4
RT	9.9	15	22.5	30	35.1

VP, vacuum pressure (in. of Hg); AT, application time (min); RT, relaxation time (min)

concentrator (Universal vacuum system, Model: UVS400-115, Thermo Electron Corporation, Milford, MA, US) and dissolving the suspension in appropriate volume of methanol. Extracts of each sample were prepared in triplicate and stored in amber vials at –70°C. Analyses of *t*-resveratrol glucoside was performed using a Waters Alliance 2695 separations module (Waters, Milford, MA) coupled with a Micromass Quattro *micro* API MS/MS system and controlled with MassLynx V4.0 data analysis system (Micromass, Cary, NC). The column used was a Phenomenex Luna C18 (150 mm × 2.1 mm, 5 μm) with a Waters X-Terra MS C18 guard column. A previously reported method [20] was modified and used for the separation of the *t*-resveratrol glucoside. A gradient elution was carried out with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.35 mL/min. A linear gradient profile was used with the following proportions of solvent A applied at time *t* (min); (*t*, A%): (0, 94%), (9, 84%), (12, 83%), (14, 83%), (16, 83%), (18, 82%), (21, 80%), (29, 0%), (31, 94%), (40, 94%). Electrospray ionization in negative ion mode (ESI–) was used for the analysis of *t*-resveratrol glucoside with the following conditions: capillary voltage 3000 V, nebulizer gas (N_2) at temperature 375°C and a flow rate of 0.35 mL/min. Multiple Reaction Monitoring (MRM) mode using a specific precursor→ product ion transition (m/z 389→227) was employed for quantification in comparison with standards of *t*-resveratrol glucoside. The concentration of *t*-resveratrol glucoside of the juice used for VI treatment was 0.12 mg/L.

2.6. Determination of Textural Characteristics

A puncture test method was performed on the dried apple slices using a texture analyzer (Model: TA.XT Plus texture analyzer, Texture Technologies Corp., New York, USA), in which a blade probe was passed through a given distance (15 mm) at the test speed of 1.00 mm/s [21]. The data were obtained for forward gradient of the deformation curve (kg/s) and linear distance traveled after contact with the sample and until rupture of the apple slice (kg.s).

3. Results and discussion

3.1. Optimization of Process Conditions for Individual Responses

The response values of *t*-resveratrol glucoside and textural attributes (gradient and linear distance) obtained by applying the central composite design using RSM are given in **Table 2**. Analysis of variance (ANOVA) was performed on each response separately **Table 3** and the results indicated that the model was adequate. There was no significant lack of fit ($p > 0.05$) which indicated that the number of experimental combinations formed in the design of experiment was enough to find out the effect of independent variables on the responses. Further examination of contour plots illustrated the relationship between experimental factors and response in two-dimensional representation generated for all the responses **Figures 1**, **2** and **3**. Multiple response optimization was done by overlaying contour plots.

Table 2. Response values for given levels of variables (vacuum pressure, application time and relaxation time) in RSM.

Experiment	Coded variables			Uncoded variables			*t*-Resveratrol glucoside (mg/100g DM)	Gradient (kg/s)[a]	Linear distance (kg.s)
				VP	AT	RT			
1	1.68	0	0	9.4	10.0	22.5	28.10	0.81	2.31
2	0	−1.68	1	6.0	1.6	30.0	24.75	0.76	2.47
3	−1	1	1	4.0	15.0	30.0	25.61	0.82	2.33
4	−1.68	−1.68	1	2.6	1.6	30.0	26.20	0.64	2.83
5,6,7,8,9,10	0	0	0	6.0	10.0	22.5	24.16	0.64	2.66
11	1	1	−1	8.0	15.0	15.0	25.79	0.63	2.65
12	−1	−1	−1	4.0	5.0	15.0	28.15	1.23	2.08
13	−1	1	−1	4.0	15.0	15.0	24.92	0.80	2.46
14	0	0	−1.68	6.0	10.0	9.9	22.56	0.59	2.46
15	0	1.68	0	6.0	18.4	22.5	25.19	0.74	2.39
16	1	−1	1	8.0	5.0	30.0	24.00	0.77	2.01
17	−1.68	0	0	2.6	10.0	22.5	22.97	0.54	2.43
18	0	0	1.68	6.0	10.0	35.1	25.45	0.58	2.78
16	0	0	0	6.0	10.0	22.5	23.58	0.67	2.45
19	1	1	1	8.0	15.0	30.0	26.24	0.70	2.79
20	1	−1	−1	8.0	5.0	15.0	18.25	0.64	2.80

[a]Gradient was transformed (X: $(1/X)^3$) before analysis to achieve normality. Untransformed values are shown in the Table. VP, vacuum pressure (in. of Hg); AT, application time (min); RT, relaxation time (min)

Table 3. Regression coefficients and analysis of variance (ANOVA).

Regression coefficients	*t*-Resveratrol glucoside	Gradient [a]	Linear distance
β_0	42.865354	–7.981650	0.832844
β_1(VP)	–4.469402	1.296660	0.076673
β_2(AT)	–0.573949	0.962972	0.147376
β_3(RT)	–0.380817	0.294952	0.062411
β_{11}	0.109618	–0.035410	0.000879
β_{12}	0.130985	0.021471	0.005405
β_{22}	0.003060	–0.039456	–0.006100
β_{13}	0.082939	–0.054076	–0.006664
β_{23}	–0.005108	–0.012119	–0.002682
β_{33}	0.000383	0.003759	0.000349
ANOVA (p-values)			
Lack-of-fit	0.0823	0.0732	0.0994

[a]Gradient was transformed (X: $(1/X)^3$) before analysis to achieve normality; VP, vacuum pressure (in. of Hg); AT, application time (min); RT, relaxation time (min)

Canonical analysis demonstrated a saddle point for all the responses examined in VI-treated apple slices **Table 4**. Also, the eigen values obtained for all the responses showed both positive and negative signs. Thus, stationarypoint for responses could not be obtained as minimum or maximum. Therefore, ridge analysis was performed to determine the levels of the design variables that would produce the maximum response for *t*-resveratrol glucoside concentration and gradient values, and minimum response for linear distance, under the given conditions **Table 5**.

3.1.2. T -Resveratrol Glucoside Concentration

The *t*-resveratrol glucoside concentration in the VI-treated, dried apple slices were used as a specific bioactive marker for the fortification using grape juice during optimization of VI conditions. The results of the canonical analysis for *t*-resveratrol glucoside concentration depicted the stationary point to be a saddle point **Table 4**. Under constant relaxation time **Figure 1(a)** and application time (**Figure 1(b)**) the saddle point can be clearly seen. The contour plots **Figure 1(a)** indicated that the incorporation of *t*-resveratrol glucoside was lower at the lower application time and vacuum pressure settings. However, above 5 in. of Hg and application time above 10 min the incorporation of *t*-resveratrol glucoside would start to increase. Similar results for higher *t*-resveratrol glucoside concentration can be obtained by keeping vacuum pressure below 5 in. of Hg and relaxation time below 25 min. When vacuum pressure is held constant (6 in. of Hg), increasing the level of application time and relaxation time would result in increased *t*-resveratrol glucoside concentration **Figure 1(c)**. The ridge analysis showed that the vacuum conditions that will maximize the uptake of this specific bioactive was vacuum pressure 8.45 in. of Hg, application time 14.45 min and relaxation time 28.11 min **Table 5**.

3.1.3. Textural Characteristics of VI–treated, Dried Apple Slices

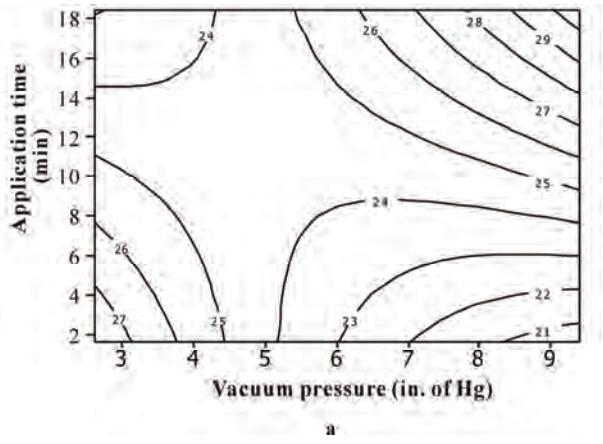
Crispiness is one of the most important textural attributes of snacks which can be explained in terms of gradient and linear distance [22,23]. In instrumental texture analysis of the VI-treated apple slices, greater values for gradient and lesser value for linear distance corresponds to higher crispiness.

3.1.3.1. Gradient

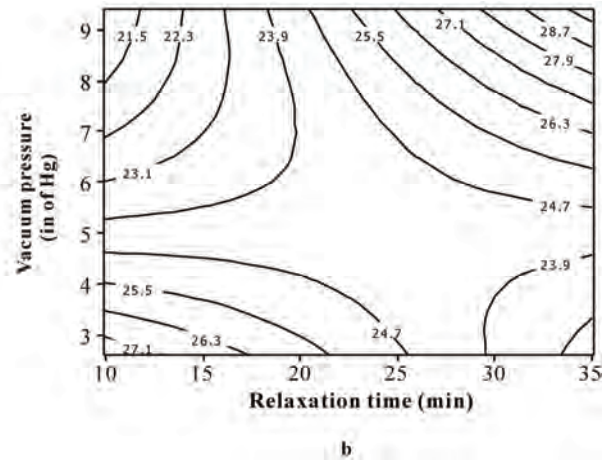
The examination of contour plots showed that VI application time had a greater impact on this textural parameter than vacuum pressure **Figure 2(a)**. The gradient value would be least (0.64 kg/s) when application time is in the range of 10 to 12 min and vacuum pressure is in the range of 4 to 9 in. of Hg. When application time is held constant (10 min) the gradient value would be affected by both vacuum pressure and relaxation time **Figure 2(b)**. Similarly, under constant vacuum pressure **Figure 2(c)**, gradient values seemed to be influenced by both application time and relaxation time. The maximum crispiness, as assessed by a large gradient value was predicted at vacuum pressure 4.04 in. of Hg, application time 8.93 min and relaxation time 32.68 min **Table 5**.

3.1.3.2. Linear distance

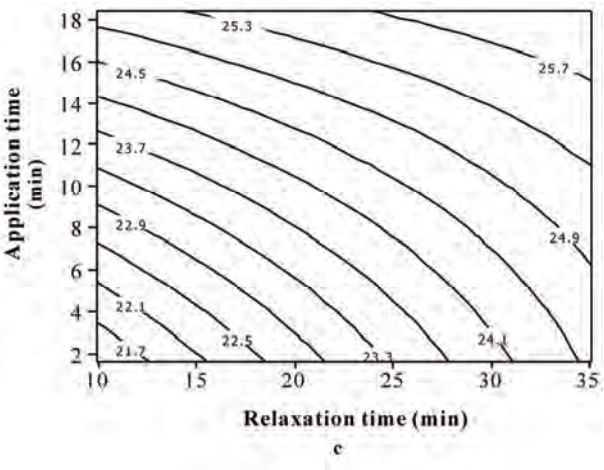
The stationary point for linear distance of dried apple slices in the canonical analysis was observed to be a saddle point which can also be seen in the contour plots **Table 4**; **Figure 3**. When relaxation time was held constant, the response values for linear distance would increase



Hold values relax. time (min) 22.5

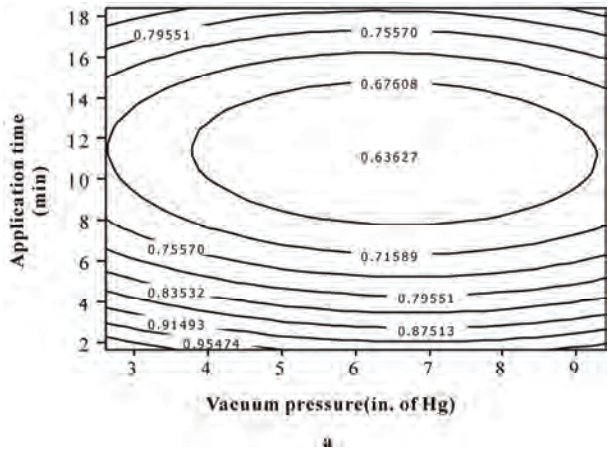


Hold Values Appl. time (min) 10

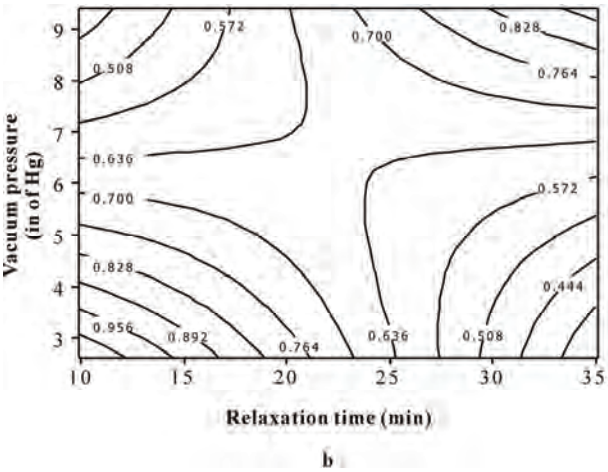


Hold values pressure (in of Hg) 6

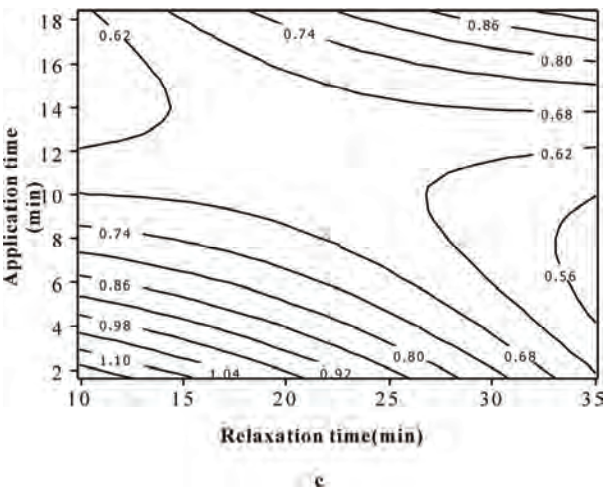
Figure 1. Contour plot of *t*-resveratrol glucoside concentration at given vacuum pressure (in. of Hg), application time (min) and relaxation time (min).



Relaxation time held constant (22.5 min)



Application time held constant (10 min)



Vacuum pressure held constant (6 in. of Hg)

Figure 2. Contour plot of gradient at given vacuum pressure (in. of Hg), application time (min) and relaxation time (min).

Table 4. Canonical analysis for optimization of VI process.

Variable	*t*-Resveratrol glucoside (mg/100 g DM)	Gradient (kg/s)	Linear distance (kg.s)
	Eigen values		
	3.24	1.47	0.22
	0.41	–1.16	–0.11
	–2.10	–2.91	–0.47
	Critical values at the coded level of variables		
VP	–0.31	–0.03	0.42
AT	–0.06	0.08	0.06
RT	0.26	–0.16	–0.04
	Critical values at the actual level of variables		
VP	4.94	5.91	7.43
AT	9.54	10.67	10.53
RT	25.77	20.47	22.01
	Predicted response value		
	24.18	0.63	2.58
	Stationary point		
	Saddle	Saddle	Saddle

VP, vacuum pressure (in. of Hg); AT, application time (min); RT, relaxation time (min)

Table 5. Ridge analysis for maximizing the individual response value in VI process.

Estimated values at coded radius 1.0	*t*-Resveratrol glucoside (mg/100 g DM)	Gradient (kg/s)	Linear Distance (kg.s)
Response	28.49	0.55	2.09
	Parameters of VI process		
VP	8.45	6.43	6.24
AT	14.45	1.78	2.19
RT	28.11	20.44	17.95

VP, vacuum pressure (in. of Hg); AT, application time (min); RT, relaxation time (min)

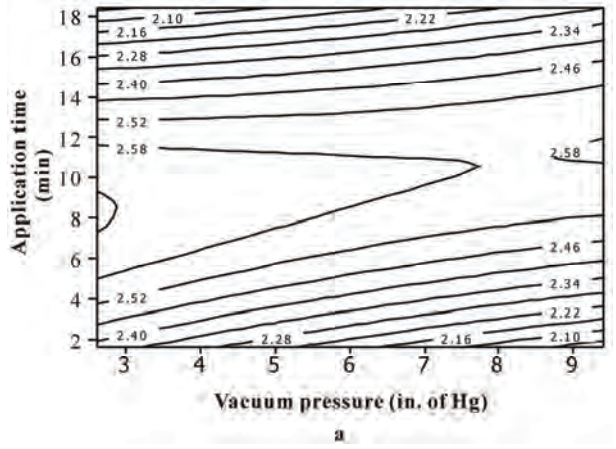
with the increasing level of application time and after reaching 12 min of application time it would start decreasing again **Figure 3(a)**. Higher levels of vacuum, within the range studied in these investigations, would result in reduced linear distance under longer relaxation time interval, and hence a more crispy product **Figure 3(b)**. Similarly, under constant vacuum pressure **Figure 3(c)**, the linear distance values would be low at the lower level of relaxation time, but would increase with application time up to certain level and then decrease after 15 min of application time. The ridge analysis was done, which showed that apple slices with minimum values for linear distance (2.09) (at a coded radius 1.0) in canonical analysis would be estimated at vacuum pressure 6.24 in. of Hg, application time 2.19 min and relaxation time 17.95 min **Table 5**.

3.2. Optimization of Process Conditions for All Responses

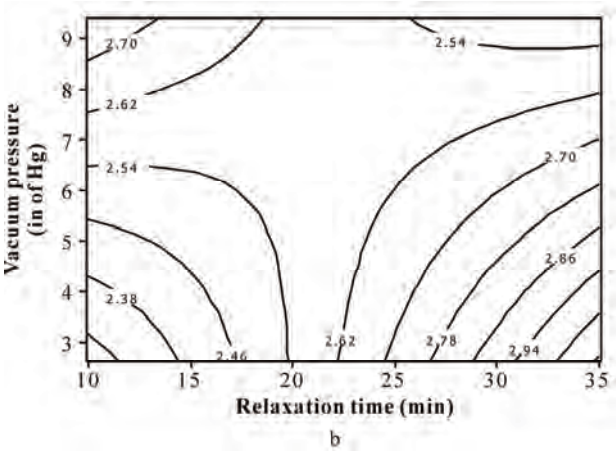
Overlaid contour plot was obtained for three responses to relate to two continuous design variables (vacuum pressure and relaxation time) while holding the third variable *i.e.*, application time in a model at 10 min **Figure 4**. Overlaying the individual plots for the responses resulted in the identification of region in the centre of the plots which satisfied all constraints as shown in **Figure 4**. Thus, based on the overlaid contour plots the optimum conditions for VI process were selected: vacuum pressure of 6 in. of Hg, application time of 10 min, and relaxation time of 22.5 min.

4. Conclusions

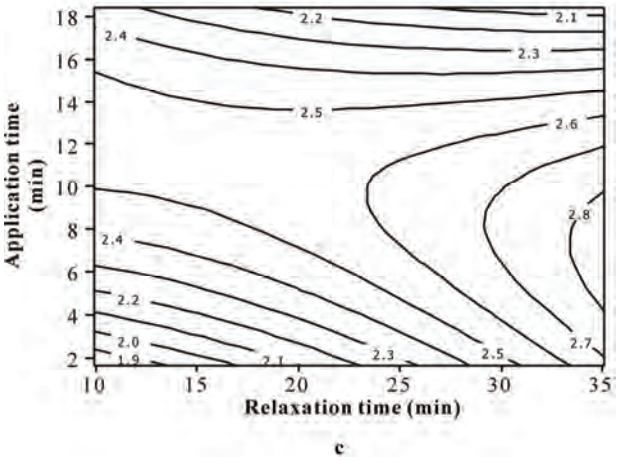
In conclusion, the VI process for vacuum pressure, application time and relaxation time can be effectively optimized using RSM for the incorporation of *t*-resveratrol glucoside, a natural bioactive from grape juice, and as well as the improvement of the textural attributes of dried apple slices. RSM was used for identifying the final optimal experimental parameters for each response individually as well as considering all responses using multi-



Relaxation time held constant (22.5 min)

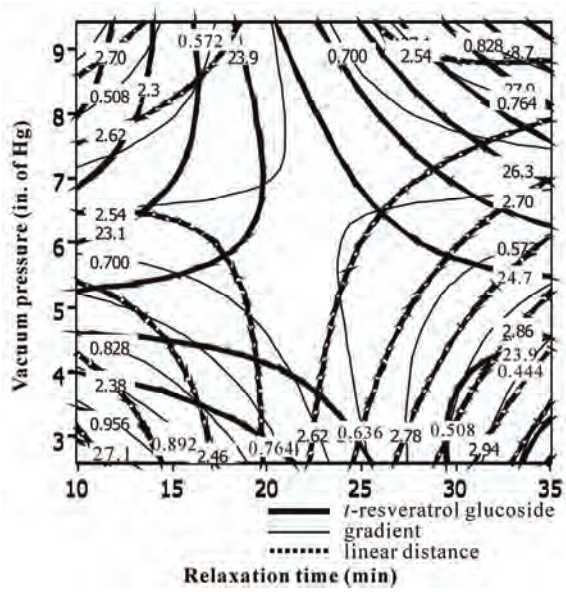


Application time held constant (10 min)



Vacuum pressure held constant (6 in. of Hg)

Figure 3. Contour plot of linear distance at given vacuum pressure (in. of Hg), application time (min) and relaxation time (min).



Application time held constant (10 min)

Figure 4. Overlaid contour plot for the optimum operation conditions.

ple response optimization. Hence, to obtain both the optimum textural quality and bioactives uptake in the apple snacks, the parameters for VI process were selected based on multiple response optimization. The developed apple snacks obtained using optimized VI-process were further compared with un-treated apple snacks for the sensory attributes, and the results are published elsewhere based on the overlaid contour plots the optimum conditions for VI process were selected: vacuum pressure of 6 in. of Hg, application time of 10 min, and relaxation time of 22.5 min.

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Bioavailability and Solubility of Different Calcium-Salts as a Basis for Calcium Enrichment of Beverages

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ABSTRACT

Background: *When usual calcium consumption patterns were analysed most people in the U.S. and Europe consume less than the recommended daily allowance. Supplements and fortified foods offer alternative and additional sources of calcium to traditional food. Calcium carbonate is the most common supplement but it is less soluble in water and, therefore, not suitable for enrichment of beverages. New organic calcium salts have a better solubility but less is known about their bioavailability. In the present study, we assessed the solubility and bioavailability of the new organic calcium salts, calcium lactate citrate and calcium lactate malate, in comparison to the traditional supplements, calcium carbonate and calcium gluconate. Design*: *Randomized, single-blind, four way cross-over study comparing single doses of* 500 *mg calcium in the form of four different calcium-salts. Subjects were advised to consume* 25 *µg vitamin D*3 *daily two weeks prior to the start of the study and during the whole study period. Subjects*: 20 *healthy young men, Methods: Blood samples were drawn immediately before and* 2, 4, 6, 8, 12 *and* 24 *h after ingestion of the calcium preparation. Concentration of total calcium and intact parathyroid hormone were measured in the serum. Urine was collected at baseline and during the intervals* 0-3, 3-6, 9-15 *and* 15-24 *h and excretion of calcium, sodium and creatinine was examined. Results*: *The tested new salts were easily water soluble, significantly better than calcium gluconate. Calcium carbonate is almost insoluble. The bioavailability of the four different calcium salts was found to be almost identical. The maximum total serum calcium increased by* 7.6 *% two hours after ingestion calcium lactate citrate, by* 7.4 *% after calcium lactate malate, by* 5.5 *% after calcium carbonate, and by* 5.8 *% after calcium gluconate. Intact parathyroid hormone concentration showed the expected depression for calcium lactate citrate, calcium lactate malate and calcium carbonate, whereas the serum level was significantly higher after ingestion of calcium gluconate. Conclusion*: *Given an almost equivalent bioavailability of the four tested calcium salts, we conclude that the new salts calcium lactate citrate and calcium lactate malate are well suited for fortification of beverages and thus to increase the average daily calcium intake.*

Keywords: *Calcium Salt, Bioavailability, Solubility, Enrichment of Beverages*

1. Introduction

Calcium (Ca) accounts for 1 to 2% of adult human body weight and is one of the major mineral components of the skeletal system. Small but essential quantities of calcium are required for nerve conductivity, muscle contraction, hormone and enzyme secretion, and blood clotting. Adequate Ca intake is essential for normal growth and development of the skeleton and teeth, and for adequate bone mineralization. In adulthood, low calcium intake has been associated with increased risk of osteoporosis and bone fractures [1,2]. The recommended dietary allowance (RDA) in adults for calcium varies between 800-1300 mg/d for adolescents, depending on the country, and 1000 mg/d for adults rising to 1200 mg/d for the elderly [2-5].

When usual calcium consumption patterns were analyzed, most population groups consumed less than the RDA. Only 32% of the US adults (exclusive of water and supplements) and 50% of the German adults (inclusive of water and supplements) met the adequate intake for calcium [1,6]. Data from NHANES (National Health and Nutrition Examination Survey) provides information on the intake of calcium from all sources, including diet and

supplement use. Thus, about 43% of Americans take calcium supplements, with an average daily intake of 331 mg/d [1,7]. Supplements and fortified foods offer alternative sources of calcium to traditional food and in many cases show an improved absorbability [8]. However, such alternative sources of calcium need to be evaluated in respect of bioavailability, solubility, cost, tolerance, convenience, taste, and compatibility with foods [9]. The most common Ca-supplement is calcium carbonate (CC) [10,11]; other available calcium salts are calcium gluconate (CG), calcium malate, calcium lactate, and calcium fumarate, or mixed salts such as calcium citrate malate, calcium lactate malate (CLM) and calcium lactate citrate (CLC).

CC is generally the least expensive and the most widely-used calcium salt. Eighty-five percent of all calcium supplements sold in the US contains calcium carbonate. CC comes in swallowable tablets and in chewable form, but its solubility is marginal and, thus, it is not suitable for fortification of fruit juices and other beverages. CG is an organic water-soluble calcium salt of gluconic acid. Data on the absorbance and bioavailability are scarce. Two studies conclude a quite similar absorption of CG to that of milk, CC, Ca acetate, Ca fumarate, or Ca lactate [12,13]. Nevertheless it is not often used to fortify beverages due to its low calcium content [11,14, 15]. CLM and CLC are mixed salts of calcium lactate with malic acid and citric acid, respectively, which offer very good water solubility. Hence, these salts can be used to fortify fruit juices and beverages.

However, no information is available concerning the the bioavailability and absorption rate of calcium from CLM or CLC. Therefore, the aim of this study was to compare the absorption of calcium after a single dose of CLM, CLC, CG, or CC in healthy male adults.

2. Material and Methods

2.1. Solubility

Firstly, the concentration of Ca in each salt and the recommended daily intake based on the Ca amount was calculated. The solubility of different calcium salts was tested in 21°C pure water and filtered apple juice (becker's bester, Germany). The maximum dissolvable amount of each salt in 100 ml water was detected producing a saturated solution of each salt (5 min shaking). The solubility tests were repeated three times in the same conditions. The amount of dissolved Ca in every saturated solution was calculated afterwards.

2.2. Bioavailability

2.2.1. Subjects

Twenty healthy male adults, aged 20 to 41 years, were recruited by advertising. The volunteers were free of chronic diseases such as bowel disease, intestinal resection, malabsorption, regional enteritis, or chronic diarrhea. None of the subjects took vitamin or mineral supplements, laxatives, anticoagulants, or other medications that could affect calcium or vitamin D serum concentrations throughout the study. Only male subjects were recruited because serum concentrations of total calcium and iPTH can be influenced by estrogens [16,17].

The study was conducted in accord with the Helsinki Declaration of 1975, as revised in 1983. The study protocol was approved by the Ethical committee of the Medical School Hannover, Germany. Informed consent was obtained from all subjects after they had received written information detailing the study and its purpose.

2.2.2. Study Design and Methods

In this randomized, single-blind, four-way crossover study, each subject served as his own control. Each subject ingested a single oral dose of one of the four different calcium preparations (CLC, CLM, CG, or CC) on four different days at two-week intervals. Each calcium salt contained 500 mg calcium and was suspended in 250 ml of filtered apple juice (becker's bester, Germany). To minimize the differences in vitamin D status and to allow optimal Ca absorption, subjects were advised to consume 25 mg of vitamin D_3 (Hevert, Nussbaum, Germany) daily, two weeks prior to the study, and throughout the whole study period. Additionally, subjects were asked to minimize dietary calcium two days before each investigation day.

The subjects fasted from 8 pm of the preceding day, except for drinking 500 ml Ca-poor water (11.5 mg Ca/l) between 7.30 and 8 pm, and 300 ml between 9.30 and 10 pm. The subjects attended our laboratory at 7.30 am. Blood samples were drawn immediately before and 2, 4, 6, 8, 12, and 24 h after ingestion of the calcium preparation. Urine was collected at the baseline and during the intervals 0-3, 3-6, 9-15, and 15-24 h. Energy expenditure-adjusted meals between 2100 kcal and 3500 kcal with low calcium and sodium content were given during the day. At 8.00 am, one of the calcium preparations was given with a standard breakfast (two slices of brown bread with spread, jam and honey, and one peach).

The primary outcome variables were serum concentrations of total Ca and intact parathyroid hormone (iPTH). Urine excretion of Ca, sodium and creatinine was examined as a secondary outcome variable. Phosphate, albumin and the amount of 25-hydroxyvitamin-D_3 (25-OH-D_3) were determined only for control in the baseline blood sample.

Total serum Ca was quantified by flame/graphite furnace atomic absorption spectroscopy [14]; iPTH in the

serum was measured by an electrochemilumines-cence-immunoassay (Elecsys, Roche, Germany); concentration of vitamin D, by an antibody based chemilu-minescent system (DiaSorin, Saluggia, Italia); concentration of serum albumin, by an immunoturbidimetric test (Roche, Eppstein, Germany); and concentration of phosphate was analyzed by an ammoniumphosphomolybdate method (Roche Diagnostics, Mannheim, Germany). The concentration of calcium and sodium in urine was quantified by flame atomic emission spectroscopy, and the excretion of creatinine was analyzed by an enzyme test (CREA plus-method; Roche Diagnostics, Mannheim, Germany).

2.2.3. Pharmacokinetic Calculations and Statistics

The maximum increase of total serum calcium concentration and the decreases of iPTH and serum phosphate for each subject and treatment group were calculated by subtracting the zero time (pre-dose) value from the maximum value observed post-dose. The increase in area under the curve (delta AUC) for serum calcium and the decrease of iPTH in area over the curve (delta AOC) were calculated using the trapezoidal rule approach.

The maximum urinary excretion of Ca was adjusted with the measured creatinine, and the ratio of calcium/ sodium was calculated. The increments in area under the curve for calcium/creatinine and calcium/sodium excretion in the urine were calculated using the trapezoidal rule approach.

Statistical analyses were performed using SPSS (statistical package for the social sciences) for Windows. The curves were plotted with Sigma-Plot for Windows. Normal distribution of the results was tested by using the Komolgorov-Smirnov-Test. All results are presented as mean ± SD. Statistical differences within and between groups were determined by paired t-test, with the significance level set at $P < 0.05$ and $P < 0.07$ as a tendency to significance.

3. Results

3.1. Ca-concentration and Solubility

The Ca-content of the four salts differed by more than fourfold. CC contained 40.0% Ca, whereas CLM is composed of 18.1% Ca, CLC of 16.2% and CG only of 9.0% Ca in one molecule. According to this, we calculated the recommended daily dose of every salt (**Table 1**).

The solubility of the four different calcium salts was tested in 21°C pure water and apple juice, respectively. **Table 1** shows the notable differences in solubility between the salts. CLM and CLC are easily soluble in water and apple juice, significantly better than CG. CC is almost water-insoluble but in filtered apple juice with a pH-value of 3.30, 1.2 mg/ml pure Ca from CC could be dissolved.

3.2. Bioavailibility

3.2.1. Absorption

The bioavailability of the three different calcium salts CLC, CLM, and CC after a single dose of 500 mg calcium was found to be almost identical (**Table 2**). Only CG seems to be slightly, but not significantly, inferior to the other three salts as measured by the total calcium serum content and the iPTH serum level 2, 4, 6, and 8 h after ingestion and calculated by the difference to the baseline.

The maximum total serum calcium increased by 7.6% 2 h after the ingestion of 500 mg calcium as CLC (+ 0.18 ± 0.15 mmol/l), by 7.4% after CLM (+ 0.17 ± 0.20 mmol/l), by 5.5% after CC (+0.13 ± 0.08 mmol/l), and by 5.8% (+0.14 ± 0.15 mmol/l) after CG. Differences between the four salts were not significant (**Table 2**). changed to negative in the CG group after 8 h, and negative amounts in the other three groups were detected after 24 h (see **Figure 1**).

Table 1. Concentration of Ca in the four different salts and solubility in water and apple juice.

Ca-salt	Ca-concentration	Quantum of salt needed for ½ RDA (500 mg Ca)	Solubility in H_2O (21°C)	Solubility in apple juice (21°C)	max. soluble Ca/100 ml H_2O	max. soluble Ca/100 ml apple juice
CLM	18.1%	2.75 g	115 g/l	110 g/l	2082 mg	1991 mg
CLC	16.2%	3.09 g	98 g/l	80 g/l	1588 mg	1296 mg
CC	40.0%	1.25 g	almost insoluble 0.014 g/l	3.0 g/l	almost insoluble 0.56 mg	120 mg
CG	9.0%	5.50 g	30 g/l	50 g/l	270 mg	450 mg

Table 2. Pharmacokinetic profile of total serum calcium after ingestion of CLC, CLM, CC, and CG.

	CC	CLM	CG	CLC
	N = 20	N = 20	N = 20	N = 20
AUC 0-2 h (mmol/l × h)	0.13 ± 0.08	0.17 ± 0.20	0.14 ± 0.15	0.18 ± 0.15
AUC 0-6 h (mmol/l × h)	0.17 ± 0.74	0.12 ± 1.93	0.21 ± 2.20	0.30 ± 2.50
AUC 0-8 h (mmol/l × h)	0.07 ± 1.28	0.24 ± 0.95	–0.36 ± 1.87	0.21 ± 0.79
AUC 0-24 h (mmol/l × h)	–1.88 ± 5.24	–0.76 ± 4.17	–1.63 ± 4.50	–1.21 ± 3.86

42.5% after CLM (–1.61 ± 0.78 pmol/l), by 42.5% after CC (–1.61 ± 0.85 pmol/l), and by 33.3% after CG (–1.28 ± 0.81 pmol/l). The differences between the four salts were not significant. However, a tendency to significance could be shown in the AOC (0-6 h) (p = 0.058). The AUCs were calculated for the periods 0-6 h, 0-8 h and 0-24 h after ingestion (see **Table 3** and **Figure 2**).

3.2.2. Urinary Excretion

Secondary outcome variables were measured in the urine. The maximum total calcium excretion amounted to 4.80 ± 2.88 mmol/l after ingestion of CC, 4.63 ± 3.25 mmol/l after CG, 4.32 ± 2.18 mmol/l after CLC, and 3.88 ± 1.64 mmol/l after consumption of CLM. We adjusted the total calcium levels with excreted creatinine and sodium in the urine relative to the intra-individual variability between the subjects.

The maximum creatinine-adjusted calcium excretion was found after ingestion of CLC (0.60 ± 0.23 mmol/l), followed by CLM (0.57 ± 0.25 mmol/l), CC (0.53 ± 0.16 mmol/l), and CG (0.47 ± 0.23 mmol/l) (not significant). Furthermore, T_{max} Ca (creatinine-adjusted) excretion was reached significantly faster after ingestion of CG (p = < 0.001), and AUC 0-6 h Ca-excretion was significantly higher after ingestion of CG (p = 0.042) compared to the other salts.

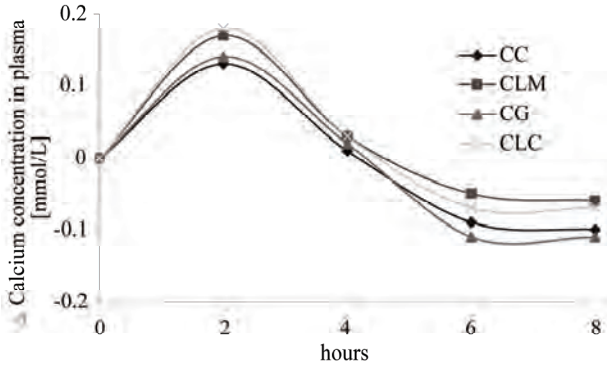

Figure 1. Time-course of the Δserum calcium for the four calcium sources over 8 h.

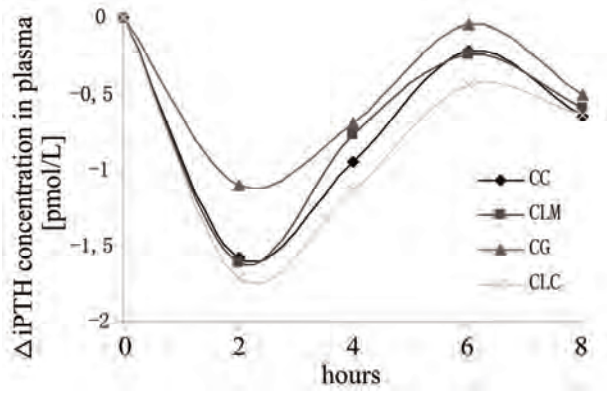

Figure 2. Time-course of Δserum iPTH concentration for the four calcium sources over 8 h.

Urinary sodium/calcium excretion (0-24 h) was comparable to creatinine-adjusted Ca excretion; the highest amount was detected after ingestion of CLC (83.8 ± 28.7 mmol/l), followed by CLM (71.6 ± 23.8 mmol/l), CG (71.4 ± 25.8 mmol/l), and CC (70.9 ± 18.0 mmol/l). The AUC 0-12 h and the AUC 0-24 h for the sodium/calcium excretion were marginally but significantly lower after the ingestion of CG compared to the other compounds (p = 0.001 and p = < 0.001, respectively).

4. Discussion

The four Ca-preparations tested induced a significant increase in serum Ca and a significant fall in serum iPTH levels, also indicating a Ca increase in young male volunteers after a single dose of 500 mg Ca. These findings are in agreement with previous papers showing significant increases in serum Ca or suppressing iPTH secretion after a single dose of Ca [11,15,18]. The current study showed a slightly superior, but not significant, increase of total serum Ca after ingestion of the two new calcium salts CLC and CLM. The suppression of iPTH was less effective after CG compared to the other three salts, and the urinary excretion maximum of calcium was lower after ingestion of CG. Therefore, we postulate a slightly better absorption of the three salts CLC, CLM and CC compared to CG.

Table 3. Pharmacokinetic profile of total serum iPTH after administration of CLC, CLM, CC, and CG.

	CC	CLM	CG	CLC
	N = 20	N = 20	N = 20	N = 20
AOC 0-2 h (pmol/l × h)	–1.61 ± 0.85	–1.61 ± 0.78	–1.28 ± 0.81	–1.72 ± 0.80
AOC 0-6 h (pmol/l × h)	–5.27 ± 3.80	–4.99 ± 4.03	–3.75 ± 3.96	–6.11 ± 3.51
AOC 0-8 h (pmol/l × h)	–6.15 ± 5.18	–5.82 ± 5.44	–4.29 ± 5.54	–7.18 ± 4.59
AOC 0-24 h (pmol/l × h)	–6.46 ± 19.95	–8.20 ± 18.41	–8.92 ± 14.03	–7.57 ± 12.08

However, Ca intake prior to the ingestion of the Ca-preparations could affect intestinal fractional Ca absorption [19]. While a high calcium intake suppresses the fractional calcium absorption, a low calcium intake stimulates the fraction that is absorbed. Dawson-Hughes *et al*. found that most of this intestinal adaptation occurs within one week [20]. Sodium intake also has important effects on the calcium metabolism [21]. To overcome the varying effects of prevailing dietary calcium and sodium intake during the four study phases, we restricted Ca and sodium intake during the whole study period and standardized the Ca and sodium intake on the four study days.

In this study we tested two new salts, CLM and CLC, which were developed as easily water-soluble salts with a rather substantial content of Ca. They were compared with two popular calcium salts, CC and CG, in water and apple juice solubility and bioavailability. CC is the most consumed calcium supplement in the US and Europe [11], but it is almost insoluble in water. We found a significantly lower solubility of CC than the other three salts even in acidic apple juice. CG is water-soluble in moderate quantities, but the content of calcium is low. It is used for people with CC intolerance and to fortify beverages. Nevertheless, aqueous solubility is only a technological advantage but not absolutely necessary for the bioavailability [15,22] because Ca needs only to be soluble in the acid medium of the stomach in order to be absorbed. However, people with achlorhydria or elderly people with reduced or absent gastric acid production have a very impaired absorption of CC when it is ingested alone [23,24]. In this case, CC can not be recommended; pH-adjusted citrate forms of Ca and water-soluble forms are a good alternative for these people. Ingesting CC together with a meal leads to an almost normal absorption in achlorhydric subjects [25].

CLM and CLC are therefore suitable for the fortification of beverages, like the established salt calcium citrate malate. Soft drinks, especially apple or orange juice which are widely consumed in the US and Europe, represent a good basis for Ca enrichment and therefore for improving the Ca intake of the population. Calcium fortification of apple and orange juices may be a potentially important vehicle for increasing the dietary intake of calcium, especially for those who do not or cannot consume dairy products. Furthermore, tests with rats revealed an almost better bioavailability of Ca from fortified juices than from milk [26]. The tolerable upper limit – the amount of calcium that can be taken during the whole lifespan without adverse effects – for calcium is 2500 mg/d [11,14]. So a fortification of 250 mg Ca per 100 ml juice would be safe and effective, assuming a consumption of one glass of fruit juice daily.

There are several limitations to this preliminary study. We carried out the study with young male subjects. Consequently, it must be shown whether the bioavailability is comparable in women and especially in elderly people. There may have been insufficient measuring points to identify the exact serum Ca-maximum and iPTH-minimum. We therefore recommend measuring points after 1-8, and 12 h for future Ca-bioavailability studies.

In summary, CLM und CLC might be seen as good water-soluble salts with a satisfactory Ca content and Ca availability comparable to the popular salts, CC and CG. We conclude that CLC and CLM appear to be a very good choice for the fortification of beverages to increase the daily Ca intake.

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Abbreviations

AUC: area under the curve, Ca: calcium, CC: calcium carbonate, CG: calcium gluconate, CLC:calcium lactate citrate, CLM: calcium lactate malate, iPTH: intact parathyroid hormone, RDA:recommended daily allowance

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Characterization of Acid-Soluble Collagen from Skins of Surf Smelt (*Hypomesus pretiosus japonicus* Brevoort)

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ABSTRACT

Acid-soluble collagen was extracted from the skins of surf smelt and characterized. The yield of collagen was high about 24.0% *on a dry weight basis. By SDS-polyacrylamide gel electrophoresis and CM-Toyopearl* 650 *M column chromatography, this collagen is a heterotrimer with a chain composition of* $\alpha 1\alpha 2\alpha 3$. *The denaturation temperature was* 32.5 ℃, *about* 4.5 ℃ *lower than that from porcine skin. Attenuated total reflectance-fourier transform infrared analysis showed that the percentage of secondary structural components in this collagen were* 11% *α-helix,* 34% *β-sheet,* 19% *β-turn, and* 21% *others. It suggests that the triple helical structure is present in the acid-soluble collagen from the skins of the surf smelt in comparison to that from the skin of porcine.*

Keywords*: *Attenuated Total Reflectance-Fourier Transform Infrared* (*ATR-FTIR*) *Analysis, Collagen, Denaturation Temperature, Skin, Surf Smelt

1. Introduction

Collagen has been extracted from the skins of vertebrate species such as pig and calf of bovine for industrial applications as functional foods, cosmetics, and biomedical materials. However, these materials cannot be used for the extraction of collagen, due to the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalophathy (TSE), foot-and-mouth disease (FMD) [1], and avian influenza which resulted in anxiety among the users of collagen and collagen-derived products from these land animals. At present, alternatives to land animals as collagen sources are extremely desired. In recent years, we tried to extract and characterize the collagens from aquatic organisms because they have been reported not likely to be associated with infections such as BSE, TSE, and FMD, thus safe collagen samples can be obtained from aquatic organisms. A high yield of collagen could be obtained from aquatic organisms, although the physical and chemical properties of these collagens were different from those of land animals [2-14]. These results indicated that collagens from aquatic organisms will have a potential to be important collagen sources for use in the food, cosmetic, and medical fields.

Surf smelt belong to the order Osmeridae which is a member of the Hypomesus family. The size of surf smelt (about 20 cm: body size; about 4%: the ratio of skin to body weight) is quite different from that of Japanese smelt (about 15 cm) that is closely related to surf smelt. Surf smelt cannot live in freshwater, however, Japanese smelt can live in both freshwater and seawater. Surf smelt live not only around Hokkaido, Japan, but also in the Kuril Islands and in the Kamchatka Peninsula. The catch of surf smelt is about 11,000 t per year by inland water fisheries from Abashiri City of Hokkaido, Japan. The Japanese consume a wide range of fish species that include tunas, prawns, shellfishes, octopi, and squids. In particular, fried fish is widely eaten as tempura in Japan. Surf smelt is one of the fish that is eaten as tempura.

In the present study, we describe the isolation and physicochemical properties of acid-soluble collagen from the skins of surf smelt for use as an alternative to mammalian collagen in the food, cosmetic, and biomedical materials.

2. Materials and Methods

2.1. Sample

Fresh surf smelt (*Hypomesus pretiosus japonicus* Brevoort) (50-65 g body weight and 15-18 cm body length) were caught in the port of Abashiri, Abashiri city, Hokkaido, Japan, and then transported in ice to our laboratory. These were stored at –85℃ until used.

2.2. Preparation of Collagen from the Skins of the Surf Smelt

All preparative procedures were carried out at 4℃ and were performed as described by Miller [15,16]. The frozen fish were thawed and the skins were descaled, trimmed of the residual fish meat, and cut out small pieces using a scalpel. The pieces (0.5 × 0.5 cm) were homogenized for 3 days with 10 volumes of 10% ethanol to remove the fat by changing the solution twice a day. The homogenate was then squeezed using cheesecloth to remove any excessive ethanol, and the residue was washed with distilled water for 1 day by changing the solution twice a day. The matter was extracted for 2 days with 0.1 M NaOH in order to remove the noncollagenous proteins by changing the solution twice a day. The matter was squeezed using cheesecloth and then washed with distilled water for 2 days by changing the solution twice a day. After lyophilization, the dried matter was used for extraction of the acid-soluble collagen. The matter was treated for 2 days with 10 volumes of 0.5 M acetic acid along with gentle stirring. The extract was centrifuged at 50,000 x g for 1 h. The supernatants were pooled and salted out to isolate and purify the collagen by selective salt precipitation; the collagen was isolated by the addition of solid NaCl to a final concentration of 0.7 M NaCl in 0.5 M acetic acid, followed by precipitation with 2.2 M NaCl in 0.05 M Tris-HCl buffer, pH 7.5. The purified collagen was obtained by centrifugation at 50,000 x g for 1 h, and the precipitate was dissolved in a minimum volume of 0.5 M acetic acid, dialyzed against 0.1 M acetic acid for 2 days by changing the solution once a day, distilled water for 3 days by changing the solution twice a day, and then lyophilized (acid-soluble collagen).

Prior to the extraction of the collagen from bones, the bones (about 0.5 × 0.5 cm^2) were broken by a hammer, and then extracted with 0.1 M NaOH for 3 days to remove any noncollagenous proteins by changing the solution twice a day. The residue was washed with chilled distilled water and lyophilized. The collagen from the dried matter was also solubilized as described above.

2.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Acid-Soluble Collagen from the Skins of the Surf Smelt

SDS-PAGE was performed as previously described [8]. The collagen (50 mg protein) was dissolved in 0.5 M Tris-HCl, pH 6.8, containing 2% SDS, 25% glycerol with 2-mercaptoethanol. After electrophoresis at 50 mA using 7.5% gel, the gel was stained with 0.25% Coomassie brilliant blue R-250 (Fluka Fine Chemical Co. Ltd., Tokyo, Japan) containing 25% ethanol and 10% acetic acid and destained with 5% methanol and 7.5% acetic acid. Molecular weight markers [myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa)] were from the Sigma-Aldrich Co. (USA) and were used as marker proteins.

2.4. Peptide Mapping of Acid-Soluble Collagen from the Skins of the Surf Smelt

The collagen samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5% SDS to easily solubilized the collagen and bolied at 100℃ for 5 min. After cooling in ice, the denatured samples were digested at 37℃ for 30 min using lysyl endopeptidase (0.24 amidase activity) from *Achromobacter lyticus* M497-1(EC 3.4.21.50; Wako Pure Chemical Industries, Ltd., Osaka, Japan). To stop the digestion, to the reaction mixture was added SDS to a final concentration of 2%, and then boiled for 5 min. The products were centrifuged at 30,000 x g for 5 min, the supernatants were pooled and used as the sample solution for SDS-PAGE using 10% gel. The molecular weight markers [myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), and soybean trypsin inbibitor (20.1 kDa)] were from the Sigma-Aldrich Co. (USA) and were used as standards.

2.5. Subunit Composition of Acid-Soluble Collagen from the Skins of the Surf Smelt

Twenty milligrams of the collagen sample was dissolved in 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea at 4℃ for 1 day by continuous stirring. The viscous solution was denatured at 45℃ for 30 min, then centrifuged at 50,000 x g at 20℃ for 1 h. The supernatants were pooled and applied to a CM-Toyopearl 650 M column (1.0 × 6.0 cm) previously equilibrated with the same buffer. The elution was performed by a linear gradient of 0-0.15 M NaCl in the same buffer at the flow rate of 0.8 ml/min. The subunit components were detected at 230 nm and the fractions indicated by the numbers were examined by SDS-PAGE using 7.5% gel.

2.6. Denaturation Temperature of Acid-Soluble Collagen from the Skins of the Surf Smelt

A Canon-Fenske type viscometer with an average shear gradient of 400 sec^{-1} containing 5 milliliters of 0.03% collagen in 0.1 M acetic acid was immersed in a water bath at temperatures of 20-50℃. After incubation at each temperature for 30 min, the viscosity of the collagen solution was measured and the fractional change was calculated as follows:

Relative viscosity = η_r = flow time of sample solution/flow time of 0.1 M acetic acid

Specific viscosity = $\eta_{sp} = (\eta - \eta_0)/\eta_0 = \eta_r - 1$

The denaturation temperature was determined as the temperature causing a 50% decrease in viscosity. Each point is the mean of triplicate determinations.

2.7. Amino Acid Analysis of Acid-Soluble Collagen from the Skins of the Surf Smelt

An amino acid analysis was performed to clarify the relationship between the contents of the imino acids and the denaturation temperature of the collagen sample. The collagens were hydrolyzed under reduced pressure in 6 M HCl at 110℃ for 24 h, and the hydrolysates were analyzed using a JASCO liquid-chromatography system by on-line precolumn derivatization with *o*-phthalaldehyde. This system consisted of a JASCO PU-2080 plus intelligent HPLC-pump, a JASCO FP-2020 plus intelligent fluorescence detector, a JASCO CO-2060 plus intelligent column thermostat, a JASCO DG-2083-53 3-line degasser, a JASCO LG-2080-02 ternary gradient unit, a JASCO AS-2057 plus intelligent sampler, and a JASCO CrestPak C18S (φ4.6 × 150 mm) reversed-phase column. The excitation and emission wavelengths were set at 345 and 455 nm, respectively.

2.8. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopy of Acid-Soluble Collagen from the Skins of the Surf Smelt

The ATR-FTIR spectra were collected at 20℃ and 40% relative humidity by coupling the ATR accessory (ATR PRO410-S: JASCO Co., Tokyo, Japan) to a JASCO (Tokyo, Japan) FT/IR-4100 type A instrument. The IR spectrometer bench was equipped with a globar source, a KBr bean splitter, and a triglycine sulfate detector. The ATR sampling device utilized a diamond internal reflection element embedded in a ZnSe support/focusing element in a single reflection configuration. The spectra were obtained over the range of 4000-650 cm^{-1} at a 4 cm^{-1} resolution. The resultant spectra were analyzed using an IR protein secondary structure analysis program (JASCO Co., Tokyo, Japan).

3. Results and Discussion

3.1. Preparation of Acid-Soluble Collagen from the Skins of the Surf Smelt

The skins from the surf smelt were treated with ethanol and followed by the removal of the noncollagenous constituents with NaOH. As a result, the yield of the lyophilized matter was about 8.6% on a wet weight basis. Next, the matter was extracted with acetic acid. As a result, collagen was successfully solubilized and the yield was approximately 24.0% on a dry weight basis (about 2.1% on a raw weight basis). In recent years, a trial has been aggressively performed to extract the collagen from the aquatic organisms to utilize it in industry and the collagens obtained from these organisms have been used in the food, cosmetic, and medical fields. In fact, our groups have reported the yields of collagens from marine organisms as follows: fish skin (Japanese sea bass, 51.4%, chub mackerel, 49.8%, bullhead shark, 50.1%, and ocellate puffer fish, 44.7%) [7,10], purple sea urchin test (35.0%) [11], fish bone (Japanese sea bass, 40.7%, horse mackerel, 43.5%, and ayu, 53.6%) [12], edible jellyfish exumbrella (46.4%) [4], rhizostomous jellyfish mesogloea (35.2%) [5], *Callistoctopus arakawai* arm (62.9%) [8], paper nautilus outer skin (50.0%) [13], cuttlefish outer skin (35.0%) [6], and common minke whale *unesu* [14], respectively. Recetly, some researchers reported the yields of fish skin collagen as follows: channel catfish acid-soluble (25.8%) and pepsin-soluble collagens (38.4%) [17], deep-sea redfish acid-solubilized (47.5%) and pepsin-solubilized collagens (92.2%) [18], and grass carp pepsin-soluble collagen (46.6%) [19], respectively. On the other hand, the acid-soluble collagen from the bones of the surf smelt was only slightly extracted and the yield was only 0.8% on a dry weight basis.

3.2. SDS-PAGE Patterns of Acid-Soluble Collagen from the Skins of the Surf Smelt

Acid-soluble collagen from the skins of the surf smelt was measured by SDS-PAGE using 7.5% gel. The two distinct bonds were indicated in the stained gel in mobility in the α region; this collagen existed as trimers consisting of two distinct α chains, such as α1 and α2, although the existence of the α3 chain was not identified under this electrophoretic condition **Figure 1**. Moreover, a small amount of the β chain was detected in this collagen. The same results were obtained in the collagen from the bones of the surf smelt. The positions of the chains of the collagen from the skins and bones of the surf smelt were similar to those of porcine skin **Figure 1**. These results indicate that acid-soluble collagens from the skins and bones of the surf smelt may have a chain composition of $(\alpha1)_2\alpha2$ heterotrimer are the type I collagen as a

major component in mammalian collagen, such as porcine.

3.3. Peptide Mapping of Acid-Soluble Collagen from the Skins of the Surf Smelt

The collagen digested by lysyl endopeptidase (originally isolated from *Achromobacter lyticus* M497-1 specifically cleaves the peptides on the carboxy-terminal side of the lysine residues) was applied to SDS-PAGE using a 10% gel to compare the patterns of the peptide fragment with porcine skin and the surf smelt bones. As a result, collagens from the skins and bones of the surf smelt were hydrolyzed to some extent by the enzyme with the nearly appearance of the peptide fragments with a molecular weight under about 80 kDa: this indicates the digestion of subunit components having a molecular weight of about 110-130 kDa by lysyl endopeptidase **Figure 2.** On the other hand, the collagen from the porcine skin was not fully digested under this condition; the protein bands showing α and β chains were detected, although there were some protein bands produced by digestion using the lysyl endopeptidase.

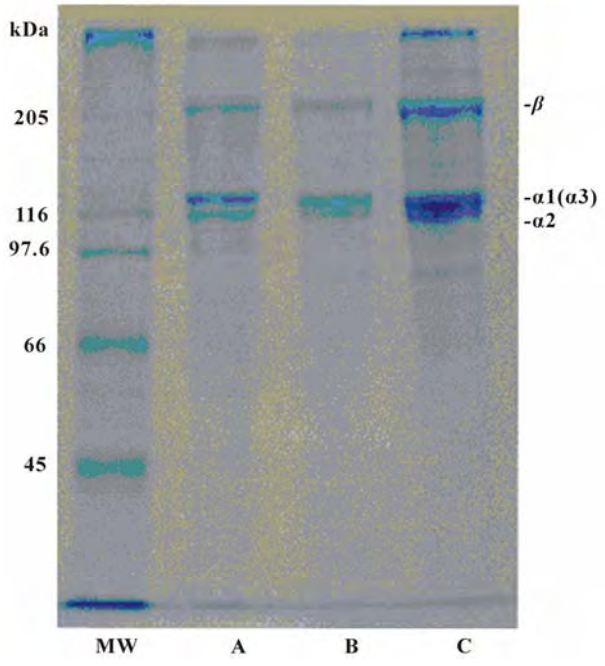

Figure 1. SDS-polyacrylamide gel electrophoresis of porcine skin type I collagen (10μg protein) and surf smelt skins and bone acid-soluble collagens (10μg protein) on 7.5% gels containing 3.5 M urea. (MW): high molecular marker: (A): acid-soluble collagen from the skins of the surf smelt; (B): acid-soluble collagen from bone of the surf smelt; (C): porcine skin collagen.

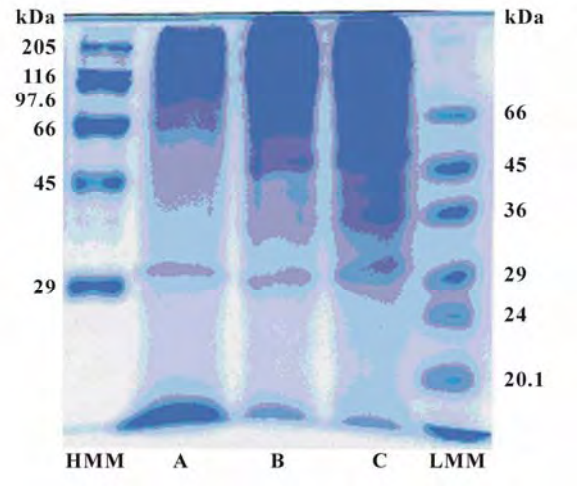

Figure 2. Peptide mapping of lysyl endopeptidase digests from collagen samples. (HMM): high molecular marker; (A) porcine skin collagen; (B) acid-soluble collagen from the skins of the surf smelt; (C) acid-soluble collagen from bone of the surf smelt; (LMM): low molecular marker. The concentration of each collagen sample was used as 20 μg protein.

3.4. Subunit Composition of Acid-Soluble Collagen from the Skins of the Surf Smelt

The collagen was denatured and then applied to a CM-Toyopearl 650M column to separate the subunit components of the acid-soluble collagen from the skins of the surf smelt. This was resolved by column chromatography into two peaks, one large and one small **Figure 3**. To identify each α chain, several chromatographic fractions as indicated by the numbers were analyzed by SDS-PAGE. These results suggest that this collagen consists of three α chains. An α1 chain was found in fractions 5-8 as indicated by the numbers. An α2 chain was found in fractions 16-21 and the α3 chain in fractions 13-19 **Figure 3**. It suggested that this collagen is a heterotrimer with a chain composition of α1α2α3. Kimura *et al.* [20] examined the fish skin collagens and reported that the α3 chain was widely distributed in teleosts such as eel, sardine, chum salmon, rainbow trout, carp, anger, Alaska pollack, cod, halfbeak, common mackerel, tilapia, red barracuda, northern dab, and file fish. In a previous paper [20,21], it was reported that the α3 chain was detected in 14 fish species of 17 teleosts. On the other hand, Nagai *et al*. reported the subunit composition of collagen from aquatic organisms using the same techniques. The results were as follows: ocellate puffer fish skin [$(\alpha1)_2\alpha2$] [7], fish bone [Japanese sea bass: $(\alpha1)_2\alpha2$; horse mackerel: $(\alpha1)_3$; ayu: α1α2α3] [12], fish scale from sardine, red sea bream,

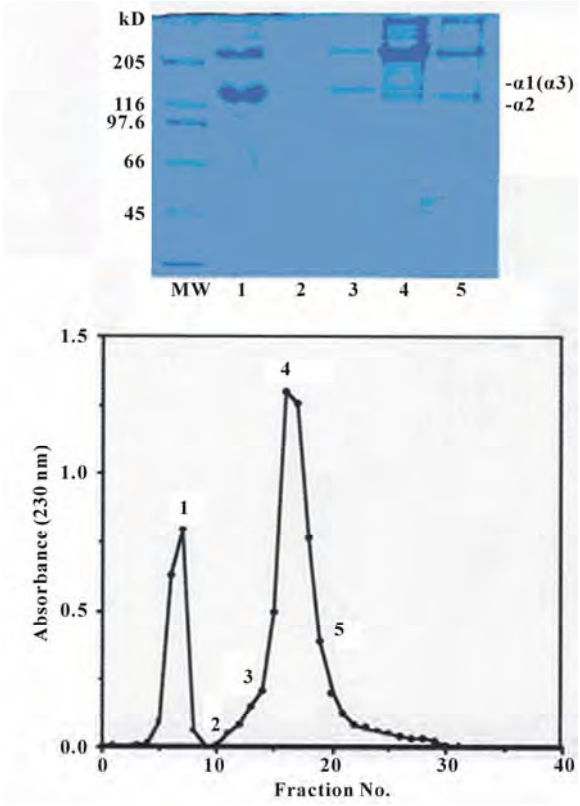

Figure 3. CM-Toyopearl 650 M column chromatography of denatured acid-soluble collagen from the skins of the surf smelt. A 1.0 × 6.0 cm column of CM-Toyopearl 650 M was equilibrated with 0.02 M sodium acetate buffer (pH 4.8) containing 6 M urea, and maintained at 37℃. The collagen sample (20.0 mg) was dissolved in 5 ml of the same buffer, denatured at 45℃ for 30 min, and then eluated from the column with a linear gradient of 0 to 0.15 M NaCl at a flow rate of 0.8 ml/min. The fractions indicated by the numbers were examined by SDS-PAGE using 7.5% gel.

and Japanese sea bass [$(\alpha1)_2\alpha2$] [9], caudul fin from Japanese sea bass [$(\alpha1)_3$] [2], purple sea urchin test [$(\alpha1)_2\alpha2$] [11], cuttlefish [$(\alpha1)_2\alpha2$] [6], diamondback squid (α1α2α3) [3], paper nautilus outer skin and *C. arakawai* arm (α1α2α3) [8]; edible jellyfish exumbrella (α1α2α3) [4], rhizostomous jellyfish mesogloea (α1α2α3α4) [5], and common minke whale *unesu* [$(\alpha1)_2\alpha2$] [14]. That is, it was obvious that the α3 chain was significantly present in the skins from aquatic organisms such as jellyfishes, cuttlefishes, and octopi.

3.5. Denaturation Temperature of Acid-Soluble Collagen from the Skins of the Surf Smelt

The denaturation temperature of the acid-soluble collagen from the skins of the surf smelt was calculated from the thermal denaturation curve. For comparison, the denaturation temperature of collagen from porcine skin was similarly measured. It was calculated that the denaturation temperature of the acid-soluble collagen from the skins of the surf smelt was about 32.5℃ **Figure 4**. This was about 4.5℃ lower than that of the collagen from porcine skin (37.0℃). This value was fairly higher than those from other aquatic organisms reported by Nagai *et al.* as follows: ocellate puffer fish skin (28.0℃) [7], fish scale from sardine (28.5℃), red sea bream (28.0℃), Japanese sea bass (28.0℃) [9], caudul fin from Japanese sea bass (28.0℃) [2], purple sea urchin test (28.0℃) [11], cuttlefish (27.0℃) [6], diamondback squid (27.5℃) [3], paper nautilus outer skin (27.0℃) [13], *C. arakawai* arm (28.0℃) [8], edible jellyfish exumbrella (26.0℃) [4],

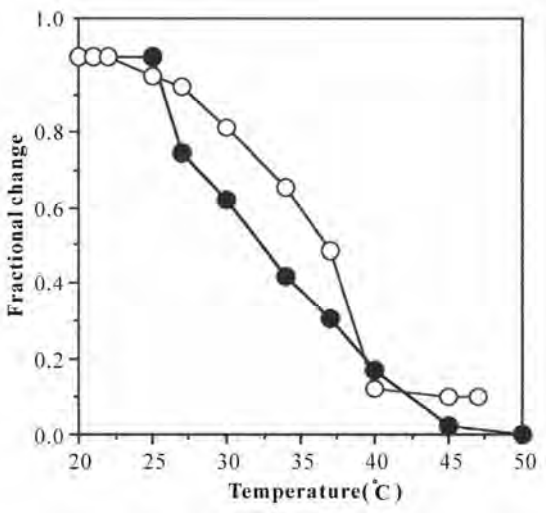

Figure 4. Thermal denaturation curves of acid-soluble collagen from the skins of the surf smelt and collagen from the skins of porcine. The denaturation temperature was measured by viscosity in 0.1 M acetic acid. The incubation time at each temperature was 30 min. Collagen concentration 0.03%; (○): porcine skin collagen; (●): acid-soluble collagen from the skins of the surf smelt.

rhizostomous jellyfish mesogloea (28.8℃) [5], and common minke whale *unesu* (31.5℃) [14]. It is well known that the denaturation temperature can be correlated with the environmental and body temperatures [22]. However, the present result was not applicable to those established theories. The reason for the high denaturation temperature of the collagen from the surf smelt is due to the difference in the hydroxylation ratio of proline, which is highly correlated to the thermal stability [23].

3.6. Amino Acid Composition of Acid-Soluble Collagen from the Skins of the Surf Smelt

The amino acid composition expressed as residues per

1000 total residues, is shown in **Table 1**. It shows that glycine was the most abundant amino acid in the acid-soluble collagen from the skins of the surf smelt and accounted for more than about one third (343 residues) among the total amino acids in this collagen. There were relatively higher contents of alanine (123 residues), proline (108 residues), and glutamic acid (74 residues), decreasing in that order. Tyrosine (3 residues), histidine (6 residues), hydroxylysine (7 residues), isoleucine (10 residues), methionine (14 residues), and phenylalanine (15 residues) were low. The imino acid, such as proline and hydroxyproine, were also detected, 172 residues: the total contents of the imino acid in this collagen were 17.2%. In previous papers, we reported the total contents of the imino acid in the collagen samples from aquatic organisms. As a result, the contents were as follows: fish scale (17.0-19.7%) [7,9], fish fin (19.3%) [2], octopus and cuttlefish skin (17.8-18.8%) [3,6,8], sea urchin test (17.9%) [11], jellyfish exumbrella (12.2%) [4], and common minke whale *unesu* (19.9%) [14], respectively. In recent years, other researchers reported the total contents of the imino acid in fish skin collagens as about 16.0-19.3% [17,19,24,25]. The degree of hydroxylation of the proline residues in the acid-soluble collagen from the skins of the surf smelt was calculated. It is well known that hydroxyproline was derived from proline by post-translational hydroxylation mediated by prolylhydroxylase, and the degree of hydroxylation was associated with the high denaturation temperature of the collagen sample. The degree of hydroxylation of the proline residues in this collagen was calculated to be about 37.2%. The previous papers showed the degree of hydroxylation of the proline residues in the collagen from aquatic organisms. As a result, the degrees of hydroxylation in collagens from fish scale [9] and fin [2], octopus [8] and cuttlefish [3,6] skins, sea urchin test [11], and common minke whale *unesu* [14] were high, but the degrees of hydroxylation in collagens from the fish skins were low [18,19,24,25]. It was suggested that the reason for not only the lower denaturation temperatures in the collagen samples from aquatic organisms is the extent of hydroxylation of imino acids, but also the lower content of the imino acid.

Table 1. Amino acid composition of acid-soluble Collagen from the skins of the surf smelt.

Amino acid	residues/1000
Hydroxyproline	64
Hydroxylysine	7
Aspartic acid	47
Threonine	22
Serine	44
Glutamic acid	74
Proline	108
Glycine	343
Alanine	123
Valine	20
Methionine	14
Isoleucine	10
Leucine	21
Tyrosine	3
Phenylalanine	15
Lysine	26
Histidine	6
Arginine	53
Total	1000

3.7. ATR-FTIR Spectroscopy Analysis of Acid-Soluble Collagen from the Skins of the Surf Smelt

The ATR-FTIR spectrum of the collagen from the skins of the surf smelt is shown in **Figure 5**. The amide A band position was detected at 3306 cm^{-1}, and it associated with the N-H stretching vibration. This indicates the existence of hydrogen bonds in the collagen sample. It is known that a free N-H stretching vibration occurs in the range of 3400 to 3440 cm^{-1}, and when the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies [26]. In other words, it suggested that more NH groups of the acid-soluble collagen from the skins of the surf smelt were involved in the hydrogen bonding. Next, the amide B band, related to the asymmetrical stretch of CH_2, was found at 2964 cm^{-1} in this collagen. The amide I band of this collagen was detected at 1640 cm^{-1}. The amide I band with its characteristic frequencies (1600-1700 cm^{-1}), mainly associated with the stretching vibrations of the carbonyl group along the polypeptide backbone [27], is a sensitive marker of the peptide secondary structure [28]. The amide II and III bands were found at 1541 and 1235 cm^{-1}, respectively. These indicated N-H bending vibrations and C-H stretching [27]. It suggested helical arrangements in the acid-soluble collagen from the skins of the surf smelt. Sarver and Krueger [29] investigated the protein secondary structure using FTIR and analyzed the resulting spectra to construct a database. To ensure that the extracted collagen was still in the native form using the secondary structure data, the theoretical value of the α-helix, β-sheet, and β-turn in the native collagen should be provided and a comparison should be made. As a result, the percentage of these components in the acid- soluble collagen from the skins of the surf smelt was 11% α-helix, 34% β-sheet, 19% β-turn, and 21% others.

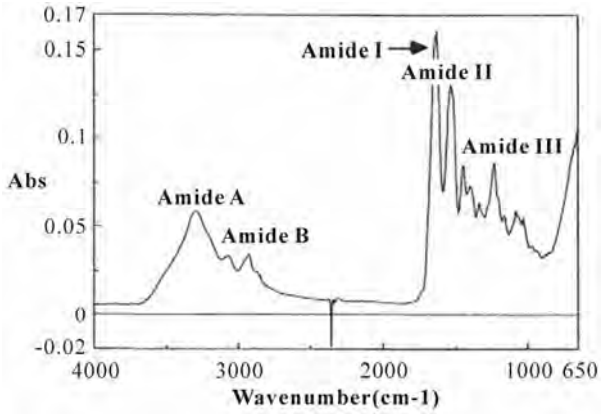

Figure 5. Fourier transform infrared spectra of acid-soluble collagen from the skins of the surf smelt.

For comparison, those of collagen from porcine skin were also calculated as follows: 9% α-helix, 51% β-sheet, 13% β-turn, and 15% others. Moreover, those of collagen from common minke whale *unesu* were as follows: 8% α-helix, 50% β-sheet, 15% β-turn, and 17% others [14]. It suggests that the triple helical structure is present in the acid-soluble collagen from the skins of the surf smelt.

4. Conclusions

Acid-soluble collagen was extracted from the skins of the surf smelt and characterized. The denaturation temperature of this collagen was high in comparison to those of other aquatic organisms. By ATR-FTIR analysis using an IR protein secondary structure analysis program developed by the JASCO Co. (Tokyo, Japan), the percentage of the secondary structural components of the collagen sample was determined. Applying this technique will have the potential to obtain new knowledge about many species of collagens.

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A Pilot Study: No Therapeutic Effect of L-Alanine in Patients with Nonalcoholic Steatohepatitis

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ABSTRACT

***Background*:** *Mitochondrial dysfunction plays a pivotal role in the progression of nonalcoholic steatohepatitis* (*NASH*). *L-alanine was shown to restore ATP content and protect the liver in various liver injury models.* ***Aim*:** *To assess the safety and therapeutic effects of long-term administration of L-alanine in patients with NASH, we conducted a pilot trial.* ***Methods*:** *Patients with NASH were enrolled and treated with* 6 - 18 *g/day L-alanine for* 12 *months and monitored for serum aminotransferases and renal function. Liver histology was obtained at baseline and* 12 *months. Changes in serum aminotransferase were assessed by differences from entry and rate of change per month using all available measures. Changes in liver histology were assessed by differences in Brunt scores of steatosis, lobular inflammation, and fibrosis.* ***Results*:** *Nine patients were enrolled and six completed the treatment. The reasons of the study withdrawal were nausea* (n = 1), *planned bariatric surgery* (n = 1), *and un-specified reason* (n = 1). *One participant experienced exacerbation of pre-existing renal failure that required hospitalization, although the medication was safely resumed after* 2*-week cessation and treatment was completed. Serum alanine aminotransferase* (*ALT*) (−24.8 ± 32.1 *IU/L vs.*0, p = 0.11) *and aspartate aminotransferase* (*AST*) (−8 ± 16.2 1*IU/L vs.* 0, p = 0.28) *were improved in* 4 *and* 3 *of the* 6 *completed participants, while rate of ALT and AST change per month showed improvement over time* (*negative slope*) *in* 5 *and* 4 *of the* 6*. Liver histology did not change significantly.* ***Conclusion*:** *The* 12*-month administration of L-alanine seems to be safe, but did not show significant therapeutic effects on serum aminotransferase or liver histology in patients with NASH, along with less than ideal tolerability.*

***Keywords*:** *Nonalcoholic Steatohepatitis, Treatment, Hepatic ATP*

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) has dramatically increased in prevalence and become one of the most common liver diseases in industrialized nations [1,2]. NAFLD includes a broad spectrum of clinico-pathological entities from simple steatosis to steatohepatitis, and cirrhosis. Nonalcoholic steatohepatitis (NASH) is one stage of NAFLD complicated with necroinflammation either with or without fibrosis. A certain portion of patients with NASH may progress to liver cirrhosis, develop hepatocellular carcinoma, or suffer a liver death [1]. Currently, no established treatments for NASH exist [3]. Therefore, effective, safe, and tolerable treatments to prevent the progression of NASH to cirrhosis are needed.

Oxidative stress is thought to play a pivotal role in NASH progression [4]. The intracellular sources of reactive oxygen species (ROS) includes various enzymes such as cytochrome P450 enzymes (e.g., cytochrome P [CYP] 2E1), NADPH oxidase, lipoxygenases, cyclooxygenases, and xanthine oxidase in addition to mitochondria. Among them, mitochondria are considered as a major source of ROS, particularly in the cells that have high concentrations of mitochondria such as heart and liver [5]. Generation of ROS occur through physiological mitochondrial oxidative phosphorylation; however, it enormously increases under mitochondrial abnormalities [5]. Functional and morphological mitochondrial abnormalities and depressed expression of functional proteins in mitochondria observed in the NASH liver suggests that oxidative stress is one of the promising therapeutic target in patients with NASH [6-8].

Previous studies showed that obese subjects, especially

subjects with NASH have impaired hepatic ATP homeostasis. Hepatic recovery following hepatic ATP depletion induced by fructose infusion become progressively less efficient as body mass index (BMI) increased and was severely impaired in patients with NASH in a study using nuclear magnetic resonance spectroscopy [9]. Since ATP supplementation is crucial for cellular functions to maintain cell viability as well as recover from injury, the impaired hepatic ATP homeostasis in NASH may also contribute the NASH progression.

L-alanine is a glycogenic amino acid used as a substrate of ATP synthesis. A series of previous animal studies showed potential therapeutic effects of L-alanine in various murine models of liver injury, *i.e.*, D-gal-induced acute liver failure, carbon tetrachloride-induced liver injury in rats, ethanol and hydrazine-induced chronic liver failure models [10-12]. The administration of L-alanine significantly increased ATP content in the liver following ATP depletion induced by D-gal when compared with control groups. L-alanine administration also enhanced gene expression related to mitochondrial function, such as subunits of F0/F1 ATPase, cytochrome C oxidase and some components of the tricarboxylic acid (TCA) cycle [11]. Furthermore, recent studies suggested that the protective effects of L-alanine are related to the restoration of ATP content [13].

To date, there are limited data from human studies [14]. However, the evidence from animal studies provided the hypothesis that the long-term administration of L-alanine may improve necroinflammation and fibrosis in patients with NASH through the augmentation of ATP and regulation of energy metabolism. Based on the rationale described above, we conducted a pilot clinical trial to assess the therapeutic efficacy as well as the safety of L-alanine supplementation in patients with NASH.

Specific aims were to assess in patients with histologically confirmed NASH 1) the safety and tolerability of long-term L-alanine supplementation and 2) the therapeutic efficacy of long-term L-alanine supplementation by evaluating liver biochemistry and histological findings.

2. Methods

Study design: This study was an open-label, single-center clinical trial conducted at Mayo Clinic, Rochester, MN. Participants received the study medication for 12 months and were followed as detailed below. The trial was approved by the Institutional Review Boards at Mayo Clinic, Rochester, MN.

Participants: Patients with histologically confirmed NASH who met entry criteria were enrolled in this study. Steatohepatitis was defined as steatosis with moderate to severe hepatocellular injury indicated by ballooning degeneration and diffuse mixed lobular inflammation with or without perisinusoidal and perivenular fibrosis, according to the Brunt classification[15]. Inclusion criteria were: 1) age between 18 and 75, 2) histological diagnosis of NASH made within one year of entry, 3) alcohol consumption of less than 20 g/day, and 4) transaminase levels [alanine aminotransferase (ALT) or aspartate aminotransferase (AST)] more than 1.5 times the upper normal limit on at least two occasions with one assessment at three months or more prior to treatment of this study. The following patients were excluded from this study: patients who had 1) any causes for liver disease other than NASH, 2) use of drugs causing steatosis-associated liver diseases within past 6 months (e.g., steroids, tamoxifen, amiodarone, and methotrexate), 3) decompensated liver disease, 4) any previous experimental treatment of NASH within the past 3 months, 5) pre-existing diseases/situations that could interfere with the results or the completion of this trial (e.g., uncontrolled diabetes mellitus, renal or liver transplant patients, and severe cardiovascular dysfunction), and 5) pregnant women. The study was conducted in compliance with the Declaration of Helsinki and approved by appropriate regulatory bodies. All patients gave written informed consent for participation.

Study medication: The therapeutic efficacy and safety of 18 g/day of L-alanine (equivalent to 0.3 g/kg/day) (Ajinomoto USA, Fort Lee, NJ) was evaluated in this trial. To ensure safety, the medication was started with a dosage of 6 g per day (equivalent amount of daily intake of L-alanine from foods in persons consuming 100 g of protein per day), then gradually increased to 18 g/day. More specifically, participants took 6 g of L-alanine (powder) once per day for the first month, twice per day (12 g/day) for the second month, then three times per day (18 g/day) from the third month for 10 months.

Baseline evaluation and monitoring: At enrollment, a complete medical history, physical examination, and laboratory assessment including serum liver biochemistries, fasting blood glucose, and lipid profile were performed. During the study period, adverse reactions and compliance were checked through phone contact at 1, 2, 3, 6, 9, and 12 months. Patients were also asked to record any symptoms, concomitant medication use, compliance, lifestyle changes, and body weight on the provided diary during the study. The participants were examined for liver histology and anthropometric measures at baseline and 12 months and serum liver enzymes [*i.e.*, ALT and AST] at baseline, 1, 2, 3, 6, 9, and 12 months. Renal function and electrolytes (blood and urine) were also assessed at baseline, 1, 2, 3, and 12 months to monitor for safety.

***Liver histology*:** The histological findings at baseline

and final evaluation were reviewed by a pathologist blinded to the patient's identity as well as to sequence of biopsy. We evaluated the following histologic features of NAFLD in this study: the grades of steatosis, lobular inflammation, and the stage of fibrosis according to Brunt criteria. [15] Briefly, steatosis was graded into 0 to 3 based on the percentage of affected hepatocytes: < 5% (grade 0), 5-33% (grade 1), 34-66% (grade 2) and > 66% (grade 3). Lobular inflammation was graded into 0 to 3 based on the numbers of inflammatory foci per 20x field: 0 (grade 0), < 2 (grade 1), 2-4 (grade 2), and > 4 (grade 3). Fibrosis was classified into 5 stages: none, normal connective tissue (stage 0), zone 3 perisinusoidal or periportal fibrosis (stage 1; 1a = mild, zone 3, perisinusoidal, 1b = moderate, zone 3, perisinusoidal, 1c = portal/periportal), moderate, zone 3, perisinusoidal and portal/periportal fibrosis (stage 2), bridging fibrosis (stage 3), cirrhosis (stage 4). For the analyses, fibrosis stage 1a, 1b, and 1c were combined and treated as stage 1.

***Statistical analysis*:** Safety of the study medication was assessed based on the proportion of patients who developed adverse reactions (e.g. symptoms, signs, illnesses, and accidents) during the trial as well as the severity of the reactions. The efficacy of the treatment was assessed by 1) changes in serum liver enzymes (ALT and AST) and 2) changes in histologic grades or stage. Changes in serum liver enzymes were calculated as differences from entry, rate of change per month in serum liver enzymes using all available measures, and as proportion of patients with normalization (and improvement) at 12 months. ALT and AST normalization were defined using the reference ranges used at Mayo Clinic (ALT: $\leq$ 55 IU/L for males and $\leq$ 45 IU/L for females; AST: $\leq$ 48 IU/L for males and $\leq$ 43 IU/L for females). Improvement of each histological finding (steatosis, lobular inflammation, and fibrosis) was defined as at least one level of improvement at 12 months. Confidence intervals for proportions were calculated using the Wilson score method. The study was designed to have 80% power to detect changes equal to 1 standard deviation (SD) in AST or ALT change with a sample size of 8. With 6 subjects, there was 80% power to detect a change equal to 1.15 SD. Statistical analyses was conducted using JMP statistical software version 7.0 (SAS Institute Inc.), R version 2.8.1 [16], and Stata version 10.1 (StataCorp, College Station, TX).

3. Results

3.1. Study Accrual and Patient's Characteristics

A total of 11 patients were enrolled in this trial. Out of the 11 patients, two decided not to participate before the study; therefore, 9 patients received the study medication. Out of the 9 patients, 3 patients were withdrawn at 5, 6, and 11 months without completing the one-year medication for the following reasons: receiving bariatric surgery and being bothered by too many urine tests (n = 1), symptom (*i.e.*, nausea) due to the medication (n = 1), and unspecified reasons (n = 1).

Baseline clinical characteristics of the study population (n = 9) are summarized in **Table 1(a)** and **1(b)**. Briefly, mean age with standard deviation was 57.4 ± 9.3 years old. Of the 9 subjects, 4 were male. Three had diabetes mellitus (stable, well-controlled), 4 had hypertension, and 4 had hyperlipidemia. Two patients had cirrhosis (stage 4).

Of the 6 patients who completed the study, 4 lost weight during the study period. Mean weight changes and % weight change at 12 months with standard deviation were −2.8 ± 5.6 kg and −1.9 ± 4.7 %, respectively.

3.2. Study Medication Compliance

Compliance information was available in 4 out of the 6 patients who completed one-year of medication: 100% compliance in two, > 98% in one, and 2 week cessation in one due to hospitalization (pre-existing renal dysfunction). The information was not available in the other 2 patients.

3.3. Safety Evaluations

There were three adverse incidence reports from 3 individuals in this study (33.3%, 95% CI:12.1%-64.6%): diarrhea in one patient who completed the study medication, nausea in another patient who requested withdrawal and renal dysfunction in conjunction with pre-existing renal disease in yet another patient who did complete the study medication after 2-week drug cessation. Otherwise, there were no reported gastrointestinal distress or adverse changes in renal function test [blood urea nitrogen (BUN), creatinine, and creatinine clearance].

3.4. Efficacy Evaluations

Changes in serum ALT and AST of all 9 enrolled patients are shown in **Figure 1** and **Table 2**. Of the 6 patients who completed the study, 5 (83%, 95% CI: 44%-97%) showed improvement in serum ALT, and 4 (67%, 95% CI: 30%-90%) showed improvement in serum AST. ALT (or AST) normalization was observed in 3 (50%, 95% CI: 19%-81%) patients. For serum ALT, mean rate of change was −1.7 (sd = 3.0) IU/L/month (p = 0.20). For serum AST, mean rate of change was −1.5 (sd = 5.5) IU/month (p = 0.30). The observed changes in serum aminotransferases were not correlated with degrees of weight changes during the study period (data are not shown).

Histological evaluation revealed that out of 6 subjects,

Table 1. (a) Patient's characteristics at baseline.

	Enrolled Subjects (N = 9)	Subjects Completing (N = 6)
Age, years old	57.4 ± 9.3	61.7 ± 3.0
Gender, Male	4 (44%)	3 (50%)
BMI, kg/m^2	34.3 ± 6.1	34.0 ± 2.7
Diabetes Mellitus*	3 (33%)	1 (17%)
Hypertension	4 (44%)	2 (33%)
Hyperlipidemia	4 (44%)	3 (50%)
ALT, IU/L	102.9 ± 66.6	93.8 ± 28.5
AST, IU/L	74.3 ± 36.0	61.2 ± 13.1
Total bilirubin, mg/dl	0.9 ± 0.6	0.9 ± 0.3
Albumin, g/dl	4.2 ± 0.5	4.2 ± 0.2
Platelet count, $x10^9/L$	207.8 ± 79.2	233.7 ± 30.1
Triglycerides, mg/dl	193.5 ± 101.8	199.5 ± 44.6
Total. cholesterol, mg/dl	180.8 ± 61.5	187.2 ± 26.6
HDL-cholesterol, mg/dl	42.0 ± 9.7	43.2 ± 4.2
LDL-cholesterol, mg/dl	100.1 ± 59.9	104.3 ± 26.2
Fasting blood sugar, mg/dl	108.7 ± 26.1	113.2 ± 11.0
Cr, mg/dl	1 ± 0	1 ± 0
BUN, mg/dl	15.6 ± 6.2	19.0 ± 1.5
CCr, ml/min	90.7 ± 20.1	92.8 ± 10.9
Histology		
Steatosis, grade	1.4 ± 0.7	1.3 ± 0.3
Lobular inflammation, grade	1.2 ± 0.4	1.2 ± 0.2
Fibrosis, stage**	2.5 ± 1.2	2.2 ± 0.5

*: Stable, well-controlled; **: There is one subject missing baseline fibrosis.

Table 1. (b) Characteristics of individual participants.

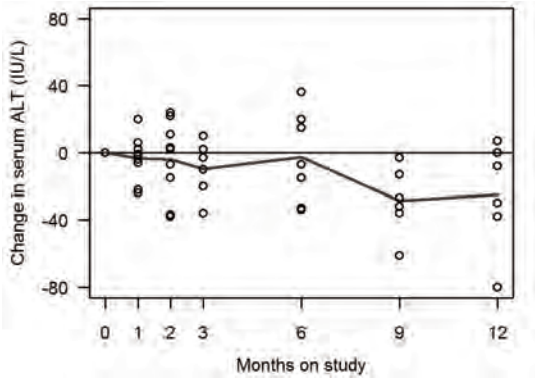
	Age	**Sex**	**DM**	**HTN**	**HL**	**Baseline histology**			**Withdrawn**
						STE	**LOB**	**FIB**	
Case 1	54.8	M	No	No	Yes	1	1	4	No
Case 2	58.6	F	Yes	No	Yes	1	1	2	No
Case 3	63.8	F	No	No	No	2	2	3	No
Case 4	54.7	M	No	Yes	No	1	1	1	No
Case 5	40.0	F	No	No	No	3	1	2	11 Mo
Case 6	71.6	M	No	No	No	2	1	-	No
Case 7	50.2	F	Yes	Yes	Yes	1	2	3	6 Mo
Case 8	66.5	F	Yes	Yes	Yes	1	1	1	No
Case 9	56.5	M	No	Yes	No	1	1	4	5 Mo

DM: diabetes mellitus; **HTN**: hypertension; **HL:** hyperlipidemia; **STE**: steatosis grade; **LOB**: lobular inflammation grade; **FIB**: fibrosis stage; There is one subject missing baseline fibrosis stage.

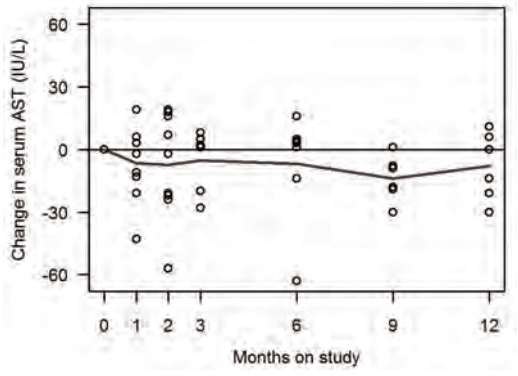
Table 2. Changes in serum ALT and AST during the administration of therapeutic dose of study medication.

	Baseline fibrosis	ALT changes			AST changes		
		At entry	Change at 12 months	Rate of change per month	At entry	Change at 12 months	Rate of change per month
Case 1	4	180	7	1.414	112	11	1.009
Case 2	2	26	0	–1.158	34	0	–0.694
Case 3	3	67	–8	–0.794	48	6	0.272
Case 4	1	154	–80	–6.840	82	–30	–2.724
Case 5	2	222	-	–1.500	112	-	–10.500
Case 6	-	81	–38	–1.911	55	–21	–0.949
Case 7	3	89	-	–5.491	130	-	–7.575
Case 8	1	55	–30	–3.386	36	–14	–1.609
Case 9	4	52	-	1.000	60	-	9.000
Mean		102.9	–24.8	–1.7	74.3	–8.0	–1.5
SD		66.6	32.1	3.0	36.0	16.2	5.5
N		9	6	9	9	6	9
P-value*		-	0.11	0.20	-	0.28	0.30

* P-values based on two-sided Wilcoxon rank sum test (vs. 0); Case 5, 7, and 9 were withdrawn at 11, 6, and 5 months, respectively.



(a)



(b)

Figure 1. Serum ALT and AST changes during the study. Changes in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) during the study are shown in Figure 1a and 1b respectively. Horizontal axis is time as months, and vertical axis is serum ALT (or AST) levels (IU/L). Each open circle in the figures represents data from individuals. Solid line represents mean values at each time point. Figures depict the changes in all the 9 participants, including 3 subjects who were withdrawn without completing the study.

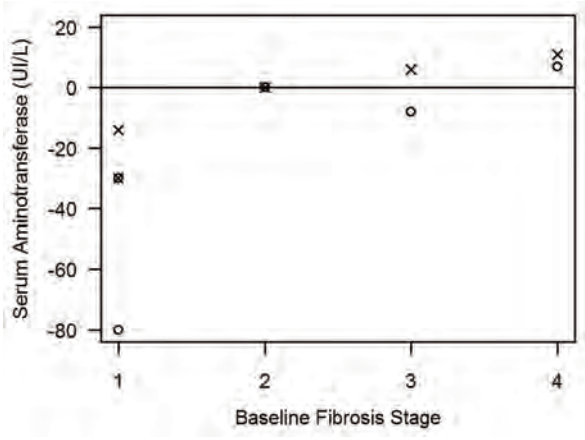


Figure 2. The associations between baseline fibrosis stages and changes of serum aminotransferase at 12 months. Figure presents the associations of baseline fibrosis stages (stage 1-4) (horizontal axis) with changes of serum alanine aminotransferase (ALT) at 12 months (IU/L) (○) and aspartate aminotransferase (AST) at 12 months (IU/L) (×) (vertical axis). The milder fibrosis at baseline was associated with greater improvement (negative value) in serum aminotransferase, while advanced fibrosis (stage 3-4) was associated with less improvement or deterioration (positive values) in serum aminotransferase.

one (17%, 95% CI: 3%-56%) showed improvement in steatosis, one showed improvement in lobular inflamma-tion, but zero (0%, 95% CI: 0%-39%) showed improvement in fibrosis.

3.5. Secondary Analysis

We further analyzed associations of the response of ALT (or AST) to the study medication with age, gender, body weight changes, baseline serum aminotransferase values,

and baseline histology. As shown in **Figure 2**, there was a positive correlation between baseline fibrosis stage and aminotransferase change at 12 months; milder stages at baseline were associated with a greater improvement (greater negative values), while severe stages at baseline were associated with less improvement or deterioration (small negative values to a positive value). This correlation still existed when using rate of aminotransferase changes per month instead (data are not shown). No associations were observed with age, gender, body weight changes during the study period, baseline steatosis or lobular inflammation.

4. Discussion

In this study, we conducted a pilot study to evaluate safety and efficacy of L-alanine in patients with histologically proven NASH. The one-year treatment with L-alanine, which was preclinically shown to restore ATP content in the liver, was acceptably safe. However, currently available data in this trial did not provide hard evidence supporting the efficacy of L-alanine in patients with NASH nor ideal tolerability. The summary statistics of serum aminotransferases showed that 4 to 5 out of 6 patients had a trend of improvement (negative slope), except for patients with advanced fibrosis. Further, the observed improvement in aminotransferases was not associated with weight loss. Thus, at this point, we cannot conclude whether there is no effect of alanine or that the insignificant results were due to the low power of this small sample to detect a beneficial effect of alanine.

Further, the study population in this study had mild inflammation at baseline; mean lobular inflammation grade was 1.2. Based on our recently published data, estimated probability of spontaneous improvement in hepatic inflammation in NASH patients was 3.0% for grade 1 and 17.8% for grade 2, and 60% for grade 3 [17], as compared with 16.7% of improvement in lobular inflammation in this study. Thus, proportion of histologic improvement observed in this study should be interpreted with caution, taking into account baseline histologic grades or stage.

Next, our secondary analysis shows that aminotransferase changes (aminotransferase response to the administration of alanine) were influenced by baseline fibrosis stage. This relationship still existed even after taking account of baseline serum aminotransferase levels (data are not shown). This relationship is opposite to "the regression to the mean", which has been observed in a historical cohort of patients with nonalcoholic steatohepatitis with two-year follow-up [17], suggesting that this association might be specifically associated with the L-alanine treatment. It would be conceivable that alanine might be effective only in patients without advanced fibrosis. However, this study cannot provide conclusive explanation for this association. Further exploration is required to delineate mechanisms explaining this possible stage-specific effect. This study has limitations. A small sample size with considerable drop-out limited our ability to assess the therapeutic effect of L-alanine with sufficient statistical power. We used liver histology as one of the study outcomes; inter-observer variance [18] and sampling error [19,20] may have affected our findings, which need to be considered in the data interpretation. Use of newly available measures (such as cytokeratin-18 fragment [21], transient elastography [22]) or use of biological or physiological endpoints (such as intra-hepatic oxidative stress, hepatic ATP homeostasis measured by nuclear magnetic resonance spectroscopy [9]) may be future consideration for assessing the effect of L-alanine.

In summary, the one-year treatment of L-alanine (up to 18 g/day) in patients with NASH was acceptably safe. This study, however, did not show significant therapeutic effects on aminotransferases or liver histology nor ideal tolerability. Considering the sampling variability, together with the above-discussed points, whether L-alanine is beneficial on patients with non-advanced NASH is still an open question. Further exploration with reconsideration of dosage and formula may be warranted.

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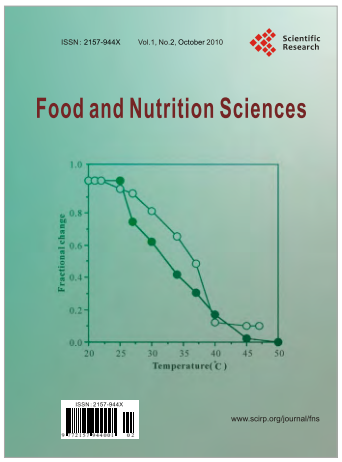

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