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Hydrothermal carbonization of microalgae II. Fatty acid, char, and algal nutrient products

Steven M. Heilmann [a,*], Lindsey R. Jader [a], Laurie A. Harned [a], Michael J. Sadowsky [a,b,c], Frederick J. Schendel [a], Paul A. Lefebvre [c], Marc G. von Keitz [a], Kenneth J. Valentas [a]

[a] BioTechnology Institute, University of Minnesota, 140 Gortner Laboratory, 1479 Gortner Ave., St. Paul, MN 55108-1041, USA
[b] Department of Soil, Water, and Climate, University of Minnesota, St. Paul, MN 55108-1041, USA
[c] Department of Plant Biology, University of Minnesota, St. Paul, MN 44108-1041, USA

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ABSTRACT

A process for isolation of three products (fatty acids, chars and nutrient-rich aqueous phases) from the hydrothermal carbonization of microalgae is described. Fatty acid products derived from hydrolysis of fatty acid ester groups in the microalgae were obtained in high yield and were found to be principally adsorbed onto the char also created in the process. With the highest lipid-containing microalga investigated, 92% of the fatty acids isolated were obtained by solvent extraction of the char product, with the remaining 8% obtained by extraction of the acidified filtrate. Obtaining the fatty acids principally by a solid–liquid extraction eliminates potential emulsification and phase separation problems commonly encountered in liquid–liquid extractions. The aqueous phase was investigated as a nutrient amendment to algal growth media, and a 20-fold dilution of the concentrate supported algal growth to a level of about half that of the optimal nutrient growth medium. Uses for the extracted char other than as a solid fuel are also discussed. Results of these studies indicate that fatty acids derived from hydrothermal carbonization of microalgae hold great promise for the production of liquid biofuels.

1. Introduction

The algal oil industry, though presently in its infancy, has tremendous potential to provide future liquid transportation fuels that can improve national energy security by providing less dependence on foreign oil. Microalgae offer significant advantages as "oil producing crops". Pound for pound, microalgae are among the most efficient plants in terms of photosynthetic activity. During growth phases they actually can double their mass, converting atmospheric carbon dioxide into carbon neutral biomass in just a matter of 3–4 h [1]. Moreover, the production of microalgae does not require high quality, arable land and therefore does not compete with food crops. Several varieties actually grow very well in salt water [2], thus providing another underutilized medium for the production of biomass. Additionally, significant quantities of lipids and lipid-derived materials can be obtained from microalgae, even exceeding half the mass of the microorganisms [3]. One report [1] predicted that a microalga having 70% oil content would require only 1.1% of US cropping area to meet 50% of all transportation fuel needs for the US, while the closest terrestrial plant, oil palm, would require 24% of the area.

While microalgae hold great promise as starting materials for biofuel production, significant challenges exist for the developing industry. Present economy of scale differences between the algal oil industry and the petrochemical industry are immense and will require significant investment in the form of government-funded incentives for liquid fuels derived from microalgae. The present microalgae manufacturing industry is very small at only 5000 tons/y [4]. It is almost completely devoted to synthesizing high value nutraceutical products and is not extensively engaged in the mass production of high oil-containing microalgae. In addition, microalgae require significantly higher levels of nitrogen than terrestrial plants to achieve effective growth rates which increases cost of production [5].

Moreover, microalgae do not achieve concentrations at maturity in their natural aqueous growth media >1 weight percent. Therefore, in order to become a significant industrial commodity in terms of cost and scale, growth and harvesting technologies need to be developed that can economically provide higher concentrations required for industrial scale processing operations. Lastly, actual extraction processes for lipids and lipid-derived materials require considerable improvement. Essentially any process suitable for commercial consideration must not dry algae by evaporating water. The energy input to evaporate water is significant, and the heat energy input required will, with very few

* Corresponding author.
E-mail address: helim001@umn.edu (S.M. Heilmann).

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exceptions, be greater than any energy output that can be obtained by combustion of the dried material.

In an investigation devoted to a mechanistic study of char formation in the hydrothermal carbonization (HTC) of distiller's grains, triacylglycerides (TAGs) were discovered to not contribute to char formation [6]. Instead, they were hydrolyzed to fatty acids under the reaction conditions and were principally adsorbed onto the char created in that process. During the preparation of this manuscript, a report appeared disclosing hydrothermal treatment of *Chlorella vulgaris* at temperatures of 225 °C and higher for 15–60 min in an unstirred condition. Residual solids were created that also contained high levels (77–90%) of adsorbed fatty acids [7]. These solids were generally not isolated *per se* but were converted directly into biodiesel fuels by a high temperature transesterification process. The present work further elaborates our initial HTC process research in which microalgae were converted into high energy char and nutrient-rich aqueous solution products [8]. The objective of the present investigation was to modify the process such that another product can be obtained, namely, fatty acids that can be transformed into liquid transportation fuels.

2. Materials and methods

Dunaliella salina was obtained as a spray-dried material from a Chinese source. *Chlamydomonas reinhardtii* [cw15 nit1-305 sta6] [9] was grown in the Bioprocess Resource Center of the University of Minnesota by a method previously disclosed [8]. A microalga was also received from Inspired Fuels, Inc. (Austin, TX). Especially with the relatively small quantities of microalgae that were available, use of freeze-dried material ensured accuracy of masses employed in experiments and efficient use of small quantities on hand. Experimental investigation as to whether aqueous slurry concentrates or freeze-dried materials gave the same results was conducting using the same lot of *Chlamydomonas reinhardtii* grown in house and harvested by centrifugation to 10% solids. A portion was freeze-dried and compared separately with fully hydrated microalga transferred into the reactor as a slurry. Identical chars in terms of % yield and %C values were isolated from HTC reactions conducted under the same conditions. This experiment indicated that freeze-drying had no affect on experimental results. FAME analyses of microalgae were conducted by Medallion Labs (Minneapolis, MN). ^{1}H-NMR analyses of product extracts were conducted using a Varian Unity Inova 400 MHz NMR with VnmrQ 2.2 Software.

2.1. Hydrothermal carbonization of microalgae

The following HTC reaction with *Chlamydomonas reinhardtii* is illustrative. Freeze-dried alga was collected from multiple preparations, ground lightly to break up agglomerated solids, placed in a container and shaken to homogenize the dry alga. Approximately 100 g of microalga were obtained and served as starting material for multiple experiments. In a typical experiment conducted at 7.5% solids, the dry alga (22.56 g) was re-dispersed in 277.5 mL of distilled water with stirring in a 450 mL stainless steel reactor. The contents were heated to 200 °C for 2 h using an induction heating system supplied by L.C. Miller, Inc. (Monterey Park, CA). After 2 h, cooling was applied in the form of a fan blowing on the unit in order to reduce the temperature of the reaction mixture as rapidly as possible. The product mixture was filtered, and the filtrate set aside. The char was thoroughly washed with distilled water and freeze-dried. The char weighed 7.05 g (31.3% mass yield based on starting alga) and was treated with three volumes of methyl t-butyl ether (MTBE). Filtration and removal of the MTBE using a rotary evaporator provided 3.30 g of a black oil and an extracted char material weighing 4.00 g. Elemental analyses of the freeze-dried chars were: 64.4%C, 8.9%H, and 6.3%N and 59.0%C, 7.4%H, and 8.8%N for the pre-extracted and extracted chars, respectively. Meanwhile, the filtrate that had been set aside having a pH of about 7 was acidified to pH 2 by addition of 8 mL of 6 N HCl. This caused precipitation of a brown solid (probably humic acids) weighing <0.2 g that did not dissolve in MTBE, benzene or hexane and was discarded. The acidified filtrate solution was extracted with an equal volume of MTBE, the organic layer was separated, and dried over anhydrous sodium sulfate. Removal of the MTBE provided 0.78 g of a light brown semi-solid. Both concentrated extracts exhibited IR spectra consistent with significant fatty acid content and were combined, providing a total yield of "extracted fats" of 4.08 g (18% mass yield based on starting alga).

2.2. NMR procedure for FAME analyses of crude oil extracts

In order to determine the actual yield of fatty acids obtained by the process, the following analytical method was devised. A reported FAME synthesis procedure using BF_3 (0.5 M) in methanol [10] was employed for the esterification, and a previously reported ^{1}H-NMR [11] procedure was modified in the following manner. In a dry Teflon-sealed vial were placed an internal standard, dimethyl terephthalate (0.0209 g; 0.0825 mmole), and the extracted fat (0.144 g) from the HTC of *Chlamydomonas reinhardtii* described above. Benzene (3 mL) was added followed by the BF_3–MeOH reagent (3 mL). The contents were sealed and heated at 85 °C in an oven for 75 min. When cool, 3 mL of water, followed by 10 mL of benzene were added. The resultant mixture was vortex mixed for 1 min and poured into a separatory funnel. The lower aqueous layer was removed and discarded. The upper organic layer was dried over anhydrous sodium sulfate, filtered, and the organic solvents removed using a rotary evaporator. The light brown oily residue weighed 0.08 g, and this residue was dissolved in 1.0 mL of benzene-d_6. A small amount of tetramethylsilane was added as an NMR reference, and a 0.10 mL aliquot was added to 0.90 mL of benzene-d_6 in a separate vial. This solution was analyzed by ^{1}H-NMR. Integration of the aromatic singlet proton resonance at 8.01 ppm for the four aromatic protons of the dimethyl terephthalate reference compound was compared to the three proton singlet resonances of the methyl esters of the FAMEs centered at ca. 3.28 ppm. Computation of the molar content of FAMEs present was possible based on the per proton resonance of the internal standard (10.00 integration units per proton for 0.0825 mmole) and for the FAME methyl ester resonances (41.3 integration units per proton for the FAMEs); the molar quantity of FAMEs present was 0.341 mmole. An approximate calculation of the mass of the FAMEs could be obtained based on the FAME analysis and the weight average molecular weight of the starting alga which was computed (284 g/mole) from the fatty acid profile provided with the Medallion analysis for *Chlamydomonas*. The molecular weight multiplied by the mmoles present in the sample provided a mass value for the FAMEs of 0.097 g. Total mass of the crude concentrated extract charged was 0.144 g, and the FAME content was 67% of product (4.08 g) or 2.73 g and 12.1% of the mass of the microalga originally charged in the reaction.

3. Results and discussion

3.1. The process

Based on our earlier observation [6] that chars obtained from distiller's grains contained significant quantities of adsorbed fatty acids and also confirmed by the recent report in which 77–90% of lipids present could be extracted from solids created during

hydrothermal treatment [7], the following procedure provided the most comprehensive process and gave the highest yields of fatty acid products. Despite constituting an additional process step and one that could negatively impact recycle of the filtrate as a nutrient for algal growth, acidification of the filtrate was included at this point because the pH of the reaction product slurry was nearly neutral. This relatively high pH may cause any fatty acid carboxylate ions present to not be extracted by the organic solvent, and a principle objective was to fully understand the total yield of fatty acids achievable and the disposition of those acids, i.e., whether on the char or in the filtrate.

The process steps adopted were:

(i) Conduct HTC of microalgae at 5–15% solids in order to minimize concentration requirements and maximize energy output scenarios,
(ii) filter the product mixture to separate filtrate and char,
(iii) acidify the filtrate to convert fatty acid carboxylate anions into fatty acids that could be extracted, and
(iv) extract fatty acid products from both char and acidified filtrate.

Proper application of the above process depended heavily on available microalgae. A characteristic of microalgae commonly observed was wide lot-to-lot compositional variation due to nutritional, harvesting, and other subtle factors. Therefore, microalgae were highly desired that could be obtained in >100 g quantities. Additionally, the lipid content of the starting microalga should be as high as possible and representative of strains that would eventually be utilized in the algal oil industry. A further important issue was the analytical determination of the lipids and lipid-derived materials obtained as an extract. It is known that various solvents give different results in extraction situations with microalgae [12]. Organic solvent extracts invariably contain components other than fatty acids such as triacylglycerides, chlorophylls, carotenoids, glycolipids, phospholipids, and sterols. Analytical methods for fatty acid (or FAME) determination were desired that were more "absolute" in their measurement. The techniques that accomplished this requirement were: (1) determination of the fatty acid content of the starting microalgae by acid-catalyzed hydrolysis, followed by FAME formation using BF_3/MeOH and gas chromatography analysis; and (2) determination of the fatty acids present in the product extract by ^{1}H-NMR analysis of the FAME content in the extract relative to an internal standard as described in Section 2.2.

The choice of the extraction solvent was also important and ether solvents that exhibited greater solubility of water were selected rather than hydrocarbon solvents because the moist filtered char containing the adsorbed fatty acids could be extracted directly rather than after drying. Methyl t-butyl ether (MTBE) was selected because of this property and its higher boiling point compared to diethyl ether.

Results obtained with three species of microalgae are given in Table 1. Each of these microalgae only partially satisfied the desired characteristics mentioned above. While *Dunaliella salina* was available in kilogram quantities, it contained low levels of lipids. *Chlamydomonas reinhardtii* was grown in house [8] and >100 g quantities were obtained, but the lipid level was moderate. A microalga obtained from Inspired Fuels, Inc. (Austin, TX) contained higher amounts of lipids but was only available in small quantities at the time of this writing.

With all three of the strains of microalgae ranging from 4% to 30% fatty acid content, the extracted fat values were consistently greater than the fatty acid values by GC and NMR because, in addition to fatty acids, other materials were also extracted, contributing to the gravimetric mass determination. It can also be stated that good correlation between fatty acid values determined by GC and NMR was achieved in all cases. With the last entry in the table having the highest quantity of fatty acids, 92% of the fatty acids were obtained from extraction of the char and only 8% from extraction of the aqueous filtrate. This latter result also indicated that acidification of the filtrate and extraction with an organic solvent were unnecessary, since excellent yield of the fatty acids could be obtained just by extracting the char. This was highly preferred because the process was simplified and no pH manipulation of the filtrate was required and no residual solvents would be present that might cause problems for sensitive growing microalgae.

3.2. Fatty acid products

In a literature report [13], the extent of complete hydrolysis of triacetin with no catalyst was determined to be 96% at equilibrium at 220 °C. Although this triacylglyceride is not a common constituent of microalgae and the reaction temperature was somewhat higher, the expectation in the present study was that the vast majority of fatty acid ester linkages would be cleaved to fatty acid products under the hydrothermal reaction conditions, although minor amounts of incompletely hydrolyzed triacylglycerides may also be present.

A significant advantage of the process was that the fatty acids were obtained from the char by a solid–liquid extraction. This type of extraction is greatly preferred to a liquid–liquid extraction that is often accompanied by emulsification and phase separation problems. Furthermore, an indication of the substantial adsorptive capacity of the chars and the quantity of fatty acids available by this extraction method was indicated by the results obtained in Table 1 with the Inspired Fuels microalga that had the highest fatty acid content (30%). Extraction of the char alone provided 92% of the isolated fatty acids and only 8% from the acidified filtrate.

The objective of the present work was primarily to determine the efficacy of obtaining fatty acid products by the HTC process. If greater purity of the fatty acid products is desired, the following purification methods can be employed:

Table 1
Results obtained with various microalgae.

Microalga	FAME yields by GC[a,b] (%)	Yields		
		Weight average molecular weight[c]	Extracted fats[a,d] (%)	FAMEs, ^{1}H-NMR[a,e] (%)
Dunaliella Salina	4	278	9	4
Chlamy-domonas reinhardtii	9	284	18	12
Inspired Fuels	27–30	290	33	28

[a] All % values are based on starting microalgae mass.
[b] Results were obtained at Medallion Laboratories, Inc., employing an acid-catalyzed hydrolysis, FAME formation using BF_3/MeOH and comparison of GC peak areas with a standard solution of FAMEs derived from fatty acids in foods [10].
[c] Values were computed from FAME profile analyses compiled by Medallion Labs.
[d] These values were gravimetric "extracted fat" masses obtained using MTBE and described in the experimental section.
[e] Internal results using dimethyl terephthalate as internal standard and methyl ester formation via BF_3/MeOH.

(1) The fatty acids in the actual extracts obtained by the process generally crystallize and can be separated by filtration, followed by washing to remove non-acidified impurities such chlorophylls, carotenoids, and sterols.
(2) Alternatively, the crude fatty acid product extract can be treated with aqueous base creating water soluble fatty acid carboxylates. Extraction of the basic aqueous solution with an organic solvent will remove all non-acidic impurities. Acidification and either extraction or filtration of solid fatty acid products will provide the fatty acids in much higher purity.

Methods for conversion of isolated fatty acids into biodiesel and green diesel fuels have been reported by application of existing refinery technology or with slight modifications [14–16].

3.3. Extracted char products

Heats of combustion of the extracted chars varied considerably depending on the fatty acid content originally present in the microalga. For strains possessing relatively high levels of triacylglycerides and related compounds, corresponding chars having especially high heats of combustion were obtained due to increased levels of adsorbed fatty acids. When these chars were extracted, the fatty acid energy component was eliminated, and the heats of combustion were reduced. With *Chlamydomonas reinhardtii,* for example, the heats of combustion of the fatty acid-containing and extracted chars were 30.11 and 24.63 MJ/kg, respectively, which was approximately a 20% reduction after extraction.

In addition to combustion of extracted chars and their use in fossil fuel replacement, other potential uses for the chars exist. The char's demonstrated ability to adsorb fatty acids indicates potential application as a hydrophobic adsorbent, for use in water and possibly air purification systems. That similar chars [17] contained a relatively hydrophobic core and yet were wetted with water due to hydrophilic functional groups such as carboxylic acids, aldehyde, hydroxyl and others present on the surface indicates that the chars may also be useful as re-enforcing additives in cement and organic polymers. The low ash content of the carbonized char [8] also suggests potential application as a carbon source for synthesis gas formation or as a coal coke alternative in steel manufacture. The material can also be stored in subterranean locations as a method of carbon sequestration or applied in soil amendment situations, since the material is fairly rapidly attacked by soil microbes (unpublished results).

3.4. Aqueous filtrate products

Some general information regarding the filtrate solution obtained from the HTC of *Chlamydomonas reinhardtii* is given in Fig. 1.

As indicated earlier, that significant quantities of fatty acids were not obtained from extraction of the acidified filtrate may prove to be very important for recycling the solution as a nutrient amendment for algal growth. The filtrate solution was completely sterile after being subjected to the 200 °C reaction condition, and its nearly neutral pH may not require adjustment. About 45% of the carbon, 80% of the nitrogen and 100% of the phosphorous were contained in the filtrate. When acidification was conducted, precipitation of a brown solid occurred, and its lack of solubility in organic solvents suggested that the solid probably was among the materials broadly categorized as humic acids having varying and complex structures [18]. There was no carbonate detected in the filtrate, and significant quantities of carbon and nitrogen were contained in Maillard-type heterocyclic compounds [19] and piperazinediones (cyclic amino acid dimers) formed in the reaction. Formation of the piperazinediones likely derived from hydrolysis

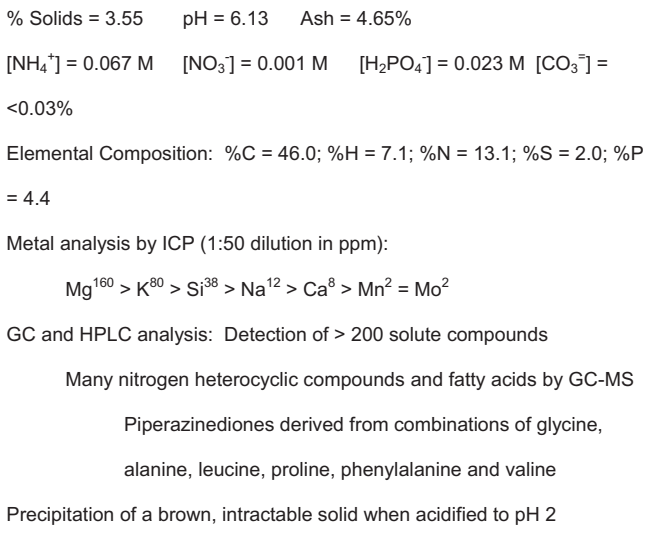

Fig. 1. Characteristics and composition of the aqueous filtrate solution from the HTC of *Chlamydomonas reinhardtii.*

of the protein structure. While there did not appear to be a general hydrolytic breakdown of the protein to amino acids under the reaction conditions, certain hydrolytically susceptible linkages may have created N-terminal amine groups on protein fragments that could displace adjacent amino acid residues via nucelophilic attack and formation of a six membered ring piperazinedione product. Some support for this conjecture and that free amino acids were not involved in piperazinedione formation under the reaction conditions was obtained from an experiment in which phenylalanine was subjected to the same reaction conditions and was isolated unchanged, and no corresponding piperazinedione was detected.

The filtrate did contain substantial quantities of potassium, nitrogen (principally in the form of ammonium ion) and phosphorous. The effect of the filtrate on an inoculum of *Chlamydomonas reinhardtii* was examined by adding varying amounts to sterilized water and compared to a control sample containing the optimum TAP medium [20]. The overall result was that by employing a ca. 20-fold dilution of the filtrate a nutrient solution was obtained that could support growth to a level of about half of that of the TAP medium. Higher levels of replacement resulted in inhibition and reduced growth. This result is preliminary and did not indicate whether additional TAP medium supplement would be required or just one or a few of its components. The effects of buildup of concentrations of Maillard heterocyclic compounds that could be toxic to algal species also require further examination. This preliminary result, however, was positive in the sense that the filtrate did function under appropriate conditions as an effective growth medium for microalgae.

4. Conclusions

A schematic representation of the HTC process for obtaining three products from microalgae is depicted in Fig. 2.

The HTC and lipid extraction process described herein has a number of advantages that could assist the advancement of the algal oil industry by overcoming some of the obstacles confronting that industry. Major advantages of the process are that algal oil products are obtained in a very simple and energy efficient manner. The process also alleviates issues regarding harvesting and production of useful co-products. Concentrations of approximately 5% are ideal for conducting the HTC process and can be achieved by microfiltration or flocculation and gravity settling techniques. Sub-

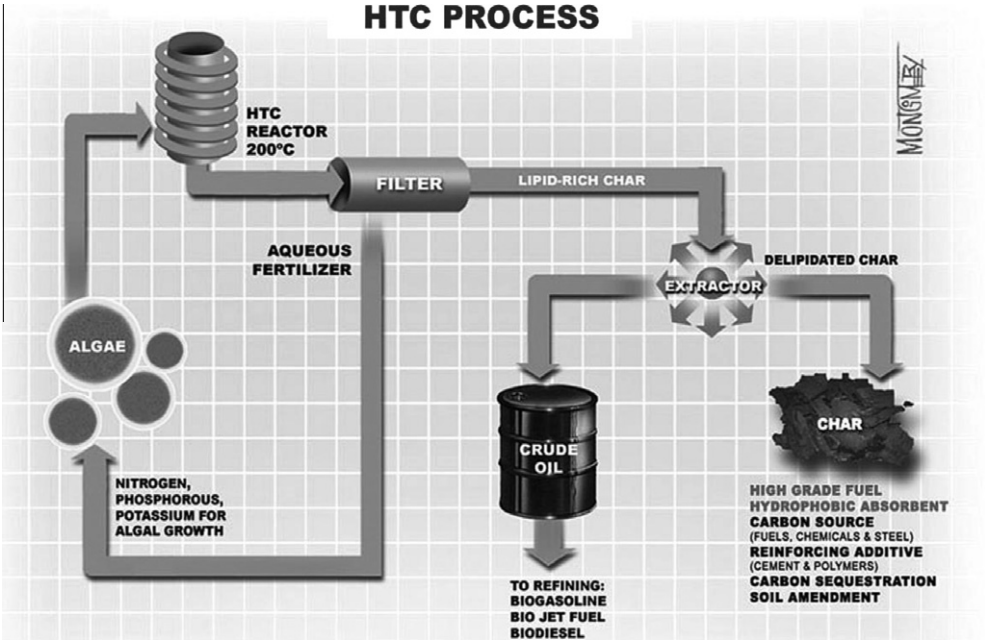

Fig. 2. Schematic diagram of the HTC process and products obtained.

stantially more expensive centrifugation operations are not required. Moreover, the most common application of extracted microalgae currently is as an animal feed. Problems associated with that application include: (1) potential toxicity issues related to extraction solvent residues and (2) with the significant increase in growth and capacity anticipated for the industry, quantities of extracted microalgae would soon swamp animal feed markets. With the HTC process, along with fatty acid products, two useful co-products are obtained—the aqueous solution filtrate that can be recycled as an algal nutrient solution and a char that has a number of demonstrated and potential uses.

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