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HPLC-MRM Relative Quantification Analysis of Fatty Acids Based on a Novel Derivatization Strategy

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Fatty acids (FAs) are associated with a series of diseases including tumors, diabetes, and heart diseases. As potential biomarkers, FAs have attracted increasing attention from both biological researchers and the pharmaceutical industry. However, poor ionization efficiency, extreme diversity, a strict dependence on internal standards and complicated multiple reaction monitoring (MRM) optimization protocols have challenged efforts to quantify FAs. In this work, a novel derivatization strategy based on 2,4-bis(diethylamino)-6-hydrazino-1,3,5-triazine was developed to enable quantification of FAs. The sensitivity of FA detection was significantly enhanced as a result of the derivatization procedure. FA quantities as low as 10 fg could be detected by high-performance liquid chromatography coupled with triple-quadrupole mass spectrometry. General MRM conditions were developed for any FA, which facilitated the quantification and extended the application of the method. The FA quantification strategy based on HPLC-MRM was carried out using deuterated derivatization reagents. "Heavy" derivatization reagents were used as internal standards (ISs) to minimize matrix effects. Prior to statistical analysis, amounts of each FA species were normalized by their corresponding IS, which guaranteed the accuracy and reliability of the method. FA changes in plasma induced by ageing were studied using this strategy. Several FA species were identified as potential ageing biomarkers. The sensitivity, accuracy, reliability, and full coverage of the method ensure that this strategy has strong potential for both biomarker discovery and lipidomic research.

Introduction

Fatty acids (FAs) play multiple critical roles in living processes: (1) FAs are critical components of the various lipid species that form biological membranes;[1] (2) as one of the most important energy compounds, FAs can supply energy through an oxidation process;[2] and (3) FAs are involved in molecular signaling and also modulate biological processes.[3] Studies have shown that FAs are associated with a variety of diseases, including tumors, diabetes, and heart diseases.[4] FA species regulate normal bioprocesses and have also been reported to influence the occurrence and development of multiple organism disorders. As potential biomarkers, FAs have attracted increasing attention both from biological researchers and the pharmaceutical industry.

HPLC-MS/MS has proven to be a powerful tool for FA quantification and has provided methods that have improved their analysis significantly.[5, 6] These methods can cover a wide range of FAs in biological samples in a single analysis.[7-9] The low ionization efficiency of FAs limit the LOQs, which have been reported to be approximately 10 pg, which is not sufficient for quantification of low-abundance FA species.[10] This situation is more pronounced in the critical analysis of biosamples. To achieve greater sensitivity, multiple strategies have been explored.[11-14] In support of sensitive detection methods, such as multiple reaction monitoring (MRM), derivatization proved to be an appropriate strategy for FA analysis.[11, 15-17] Bollinger et al. observed that the detection sensitivity of FAs was enhanced markedly with N-(4-aminomethylphenyl) pyridinium (AMPP) derivatization.[18, 19] Laborious and time-consuming optimization of MS/MS conditions is another challenge for FA analysis. Parameters such as collision energy should be optimized using standards for each FA individually prior to data collection. However, the lack of adequate standards as a result of FA diversity, which results from differences in their chain length, degree of unsaturation, double-bond position and branching structures, poses a dilemma for FA detection by MRM. Although MRM has improved the sensitivity and selectivity in FA quantification, its further application remains restricted by the lack of standards.

Researchers developed relative FA quantification strategies to elucidate the functions of FAs in various organisms.[20, 21] Matrix effects caused by complicated biofluids were a major impediment to establishing a reliable and accurate relative quantification strategy. The use of internal standards (ISs) that perform similarly to the analytes of interest proved to be an efficient approach. Isotope standards were believed to be one of

the best choices because of their chemical and physical similarity to the analytes.[22, 23] However, several obvious disadvantages of the isotope-based IS approach were observed: (1) the application of isotopic standards to the synthesis of every interested FAs is labor-intensive and expensive, and (2) this approach is limited to FAs whose structures are known. The analysis of biosamples containing diverse FA species is a significant challenge for this method.

In this study, a new derivatization strategy based on 2,4-bis(diethylamino)-6-hydrazino-1,3,5-triazine was adapted to the relative quantification of FAs by HPLC-MRM. Tagged with 2,4-bis(diethylamino)-6-hydrazino-1,3,5-triazine in mild condtions, FAs ionization efficiency was optimized sharply. As a result of this derivatization approach, we achieved an ultrasensitive FA detection method. The LOD for FAs is as low as 10 fg. Conditions are defined that result in a promising general MRM procedure for FA analysis of biological samples, which guarantees complete coverage. Using an IS with "heavy" tags containing 20 deuterium atoms, we developed an accurate and reliable FA relative quantification method. FAs in plasma samples were analyzed using this new strategy, and several FA species related to the ageing process were identified.

Experimental

Materials

N-Hydroxysuccinimide (NHS), 1-ethyl-3-[3-dimethylamino--propyl]carbodiimide hydrochloride (EDC), and all fatty acid standards(arachidic acid, arachidonic acid, behenic acid, heptadecnoic acid, myristic acid, and palmic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ceramide (18:1/10:0) and ceramide (18:1/2:0) purchased from Avanti Polar Lipids (Alabaster, AL, USA) were introduced as internal standards for pretreatment. Both "normal" and "heavy" derivatization reagents(2,4-bis(diethylamino)-6-hydrazino-1,3,5-triazine and d_{20}-2,4-bis(diethylamino)-6-hydrazino-1,3,5-triazine) were kindly provided by Prof. Zhang.[24] All solvents were HPLC grade or higher.

Methods

Rat cultures

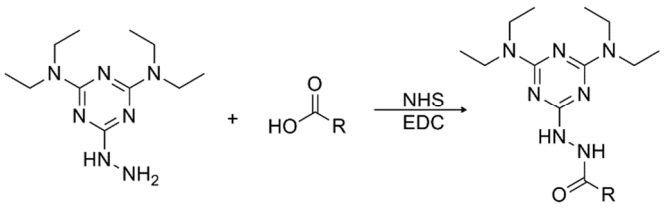
Ten male Wistar rats (8 weeks old, body weight = 200 ± 5 g) were purchased from Beijing Weitonglihua Experimental Animal Technology Co., Ltd. Rats were divided randomly into two groups. One group of 4 rats (8 weeks old) were anaesthetized with diethyl ether, and their blood was collected from the abdominal aorta. Rat blood samples were centrifuged at 4500 rpm for 15 min to obtain plasma samples. Another group containing 6 rats were cultured for another 2 months with a normal diet. Their plasma was collected following the same protocol. All plasma samples were stored at -80°C. Research was conducted in accordance with all institutional guidelines and ethics and was approved by the Laboratories Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College.

Biosample pretreatments

Ten microliters of plasma was mixed with 99.0 μl of methanol and 1.00 μl of IS, which was included to evaluate the extraction efficiency (ceramide (18:1/10:0) for "normal" tagged samples, ceramide (18:1/2:0) for "heavy" tagged samples). The samples were then vortexed for 30 s. To the mixture was added 990 μl of water, and the resulting mixture was vortexed for 30 s. The solution was loaded onto a Waters Oasis SPE cartridge (Milford, MA, USA) pre-equilibrated with 1.50 ml of methanol and 5% methanol. The cartridge was washed with 2.00 ml of 5% methanol. A 1.00 ml methanol elution sample was collected and dried. The dried sample was stored at -20°C before derivatization.

Fatty acid derivatization

The derivatization mixture was composed of 2 mg of NHS, 2 mg of EDC, 0.5 mg of derivatization reagent and 500 μl of DMSO. The derivatization mixture (5 μl) was added to each dried sample, and the solution was incubated at 37°C for 1 h for derivatization. The derivatization mechanism is shown in Scheme. "Heavy" tagged samples were derivatized following the same protocol using isotopic derivatization reagents. Prior to HPLC-MRM analysis, "normal" and "heavy" tagged FAs were mixed in equal proportions. The mixture was subjected to HPLC-MS analysis without further purification.



Scheme. FAs derivatization mechanism. Carboxyl groups of FAs were converted to hydrazides with NHS/EDC in DMSO.

HPLC–triple-quadrupole mass spectrometry analysis

An Agilent 1200 HPLC system and a 6410 triple-quadrupole mass spectrometer were used to analyze the FA derivatives. A Shiseido 50 × 2.0 mm2, C-8 reverse-phase column (Yokohama-shi, Kanagawa, JP) was used for the separation. Elution was initiated with 50% mobile phase A (acetonitrile) and 50% mobile phase B (0.2% formic acid aqueous solution). Mobile phase A was increased to 99% over a period of 7 min and held constant for another 7 min. The elution condition was returned to the initial state over a period of 1 min. The flow rate was set at 0.5 ml/min. The injection volume was 1 μl. The spray voltage was 5000 V, the nebulizer gas pressure was 35 psi, the dry gas temperature was 300°C and the flow was maintained at 11.0 l/min. The mass range was set from 20 to 700 m/z.

To adjust the MRM conditions, free and FA derivatives were prepared in 75% acetonitrile aqueous solution containing 0.2% formic acid and were subsequently injected into the mass spectrometer using a syringe pump at a flow rate of 10 μl/min. The ions pairs, collision energy, and the fragmentor were optimized automatically. The ion pairs monitored during plasma sample analysis are listed in SI.1.

Data processing

The peak areas of "normal" and "heavy" tagged FA derivatives were normalized using ceramide (18:1/10:0) and ceramide (18:1/2:0), respectively, to minimize the influence of extraction. A new value of R for each FA was generated by dividing the "normal" FA derivative peak areas by their corresponding "heavy" peak areas. The R was applied to the OPLS-DA by Simica software (Sudbury, Ontario, Canada).

Results and discussion

Mild derivatization

Elimination of double bonds leads to protection of unsaturated FAs against oxidation. Critical conditions could result in FA

degradation. To achieve mild derivatization, we tested a series of conditions. As shown in Fig. 1, the derivatization reaction was completed within 1 h at 37°C. More than 90% FA was converted into hydrazides (SI.2). Higher temperatures resulted in higher derivatization rates, and the reactions were completed in less time; however, higher yields were generated at lower temperatures. Higher temperatures and longer reaction times did not improve the derivatization yields. We attributed this optimization to the hydrazine groups of the labelling reagents. Nitrogen atoms in hydrazine are highly nucleophilic, which accelerates the reaction and results in milder conditions. With decreasing reaction temperature and time, the risk of oxidation was minimized. The low temperature inhibits the degradation and leads to higher final yields. This optimized derivatization procedure is suitable for highly unsaturated FA analysis.

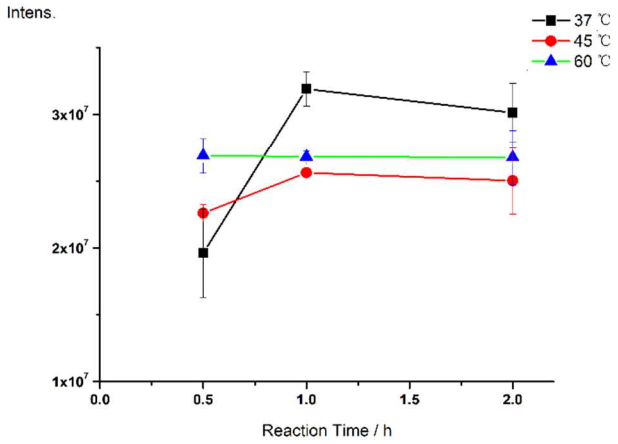


Fig.1 Myristic acid derivation completion vs. reaction time and temperature. Different lines indicate different reaction temperatures (black: 37°C; red: 45°C; blue: 60°C). No obvious advantage of increased temperatures or times was observed.

Optimization of chromatographic performance by derivatization

The highly hydrophobic nature of FAs promotes strong retention on reverse-phase columns, resulting in extended retention times and significant peak broadening. These problems impede the efficiency and sensitivity of FA analysis. The derivatization strategy was designed to solve these problems. The tag containing four protonated nitrogen atoms was adapted to FA derivatives, which significantly increased the FA derivatives' hydrophilicity. As shown in Fig. 2, FA analysis can be completed in 10 min, which is less than one-third the time required for separation of free FAs.[25] In addition, the peak widths were all within 0.2 min. These observations indicate that derivatization improved both the separation speed and the peak widths in FA analysis. The time, labor, and amount of solvent required for FA analysis was reduced by derivatization, which promises to be a high-throughput and environmentally friendly strategy for FA analysis.

Enhancement of sensitivity by derivatization

HPLC-MS analysis of FAs is prone to low sensitivity due to poor ionization efficiency. In the analysis of biosamples, especially some critical samples, poor sensitivity becomes the most essential analytical challenge for both FA qualification and quantification. Our derivatization attempts were intended to enable low-concentration FA analysis. In previous integrated analysis, derivatization strategies proved capable of achieving these goals. When reacted with hydrazine, the carboxyl groups in the FAs are converted to hydrazides. Both the hydrophobicity and the alkalinity of the FAs, which are important in the

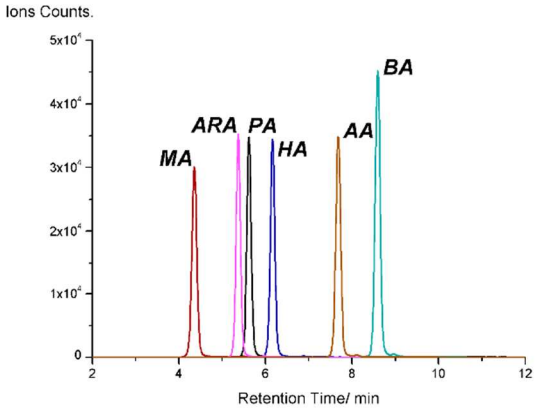


Fig.2 Derivatives of FA standards were analyzed by HPLC-MRM. With hydrophilic tags, the FA chromatographic separations were optimized. A single analysis was completed in 10 min with good resolution.

ionization process, were improved. The FA derivative ionization efficiency was enhanced, resulting in greater sensitivity. To study the sensitivity enhancement afforded by derivatization, a mixture containing different FAs at concentrations of 100 ng/ml was divided equally into two tubes and analyzed with and without derivatization. Both free FAs and FA derivatives were analyzed by HPLC-MRM. As shown in Fig. 3, the MS response for FA derivatives was approximately one thousand times greater than that for free FAs. With derivatization, FA quantities as small as 100 fg could be detected, whereas no less than 100 pg of free FAs were detected by HPLC-MRM. Other standard FAs with different structures were analyzed as well. Because of differences in the structures of FAs, derivatives LODs from 10.0 pg/mL to 1.00 ng/mL were achieved. 1000-fold improvement of the sensitivity was observed for the derivatized FAs compared to that for the free FAs (as shown in SI.3). This ultra-sensitivity enables higher coverage for FA profiling, which is important for both biomarker surveys and metabolomics research.

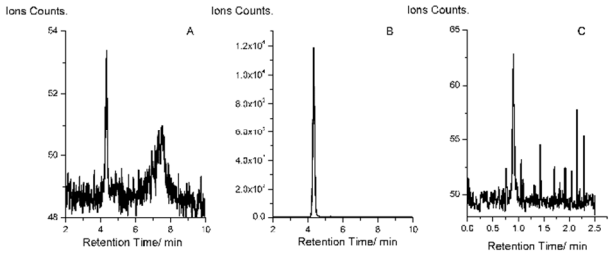


Fig.3 Enhancement of sensitivity by derivatization: 100 pg MAs derivatives (B) produce approximately a 1000-fold greater MS response than free MAs (C). This improved response reduced the FA LOD to 100 fg (A) with a S/N ratio of 3. The LODs of other FAs were enhanced by derivatization as well (SI.3). MRM conditions were shown in Table. 1

General MRM conditions applicable to any FA

HPLC-MRM was adapted for FA analysis with high specificity and sensitivity. However, the specific MRM conditions optimized for standards restricted the MRM analysis of FAs of unknown structure. The extensive amount of time and labor required for this optimization also applies to the analysis of FAs. The situation was reversed by derivatization. Fragments related to tags could be generated from any FA derivative, as shown in SI.4. We developed a general set of conditions for MRM analysis for all FAs using the same hydrazide bond connecting tags and FAs. To test our hypothesis, we optimized the MRM parameters for six different FA derivatives individually. The optimized collision energy and fragmentors are listed in Table.1. The collision energy and fragmentor were kept constant for all six of the investigated FAs. Neither the alkyl chains nor the degree of unsaturation in the FA structures influenced fragment generation. This establishes the feasibility of applying our approach of general MRM condition to the analysis of all FAs. The general MRM condition removes the dependence on specific MRM condition optimization for FA analysis. The coverage of FAs is no longer limited by the availability of standards. The HPLC-MRM method could, theoretically, fully cover all the FA species present in samples.

Table 1. FA derivatives' MRM parameters

FAs	m/z		RT/min	MRM Parameters	
	Parent ions	Fragments		CE/eV	Fragmentor
AA	548	254	7.72	40	200
ARA	540	254	5.37	40	200
BA	576	254	8.60	40	200
HA	506	254	6.22	40	200
MA	464	254	4.36	40	200
PA	492	254	5.61	40	200

AA: arachidic acid; ARA: arachidonic acid; BA: behenic acid; HA: heptadecanoic acid; MA: myristic acid; PA: palmitic acid

Establishment of an accurate and reliable relative quantification approach

As with other lipids, matrix effects hinder FA quantification and diminish accuracy and reliability. In lipidomic studies, several ISs are spiked for each class of compound to enable identification and quantification. This one-per-class method proved to be efficient and accurate.[26] Although the same challenges were encountered in FA analysis, neither efficiency nor accuracy of this one-per-class strategy performed as expected. As shown in Fig. 2, considerable differences in signal strengths were observed in five saturated-chain FAs of the same concentration. Inaccuracy resulting from their ionization efficiency would be inevitable if the one-per-class IS method was applied. These differences in MS responses were attributed to different ionization efficiencies induced by elution compositions. Increasing the organic solvent ratio decreased the surface tension of the eluent and consequently generated more gas-phase ions of analytes. To establish accurate quantification methods, we developed a parallel IS-based relative quantification strategy based on isotopic derivatization reagents. In "heavy" tags, twenty hydrogen atoms were replaced by deuterium atoms, which generated a 20 Da mass shift. Pooled samples were labeled with "heavy" tags and spiked into "normal" tagged analytes as parallel ISs. The mixture was analyzed by HPLC-MRM. As shown in Fig. 4, the close retention times of "heavy" tagged ISs and their matching "normal" tagged FAs guaranteed similar elution for IS–analyte pairs. This ensures that an IS–analyte pair would be ionized under similar conditions. With normalization by their matching IS, the quantification data for each FA was entirely related to their abundance. In this way, the accuracy of this relative quantification method was assured.

To analyze the capability of this relative quantification method, mixtures containing different ratios of "normal" and "heavy" tagged FAs (1:10, 1:5, 1:1, 5:1, 10:1) were mixed and analyzed using the relative quantification strategy. The observed ratios were plotted as functions of the expected ratios (SI.5). The relative quantification strategy showed good agreement with the expected ratio. Comparison between the relative quantification and external standard quantification were carried out with BA. Relative quantification obtained similar results to external standards quantification (SI.5). With full consideration of the derivatization conditions, coverage, and quantification accuracy, the FA relative quantification strategy has proven to be a powerful tool for FA analysis.

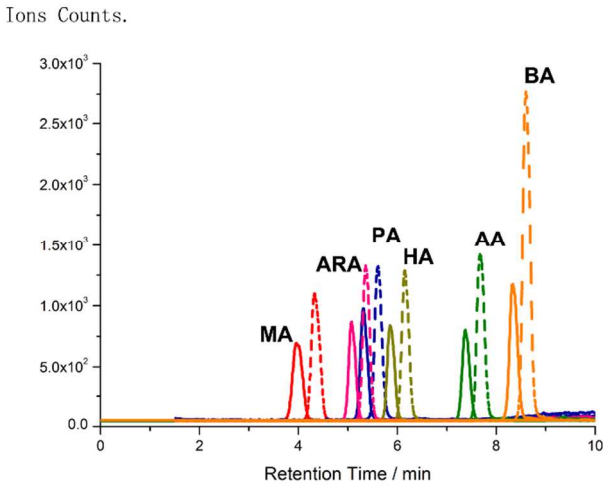


Fig.4 Relative quantification with "heavy" tagged FAs as internal standards. "Normal" (solid lines) and "heavy" FA derivative (dotted lines) mixtures were analyzed by HPLC-MRM. Parallel FA derivatives are indicated by the same color. Their retention times are similar, which indicates that they experienced similar matrix effects.

Discovery of FA species related to the ageing process

The FA analysis method was applied to study FA compositional changes in plasma related to ageing. Two groups of rats were involved: one group was 8 weeks old, and the other group was cultured for 2 additional months. Fifty microliters of plasma was collected from all of the animals and was combined and treated with isotopic reagents to form a parallel IS for relative quantification. All others were treated with normal reagents and mixed with equal volumes of IS prior to HPLC-MRM analysis. More than 30 FA ion pairs (shown in SI.1) were monitored and quantified successfully. As shown in Fig.

5A, the FA compositions changed after 2 months of ageing. On the basis of the quantification data, a T-test was performed to determine the most divergent FA species between the model and control groups. FA species whose significance was less than 0.05 were analyzed by OPLS-DA. As shown in Figs. 5C and D, the aged rats and young rats could be distinguished on the plot. Aged rats did not cluster as closely as young rats, which we believed to be related to the different health homogeneities of the two groups. The aged rats were more likely to carry different diseases along with the culture, whereas the health conditions of the young rats were very similar. These differences resulted in a different distribution of the spots. As shown in Fig. 5B, the amounts of several FA species changed dramatically between the two groups; these species can be regarded as potential biomarkers for the ageing process.

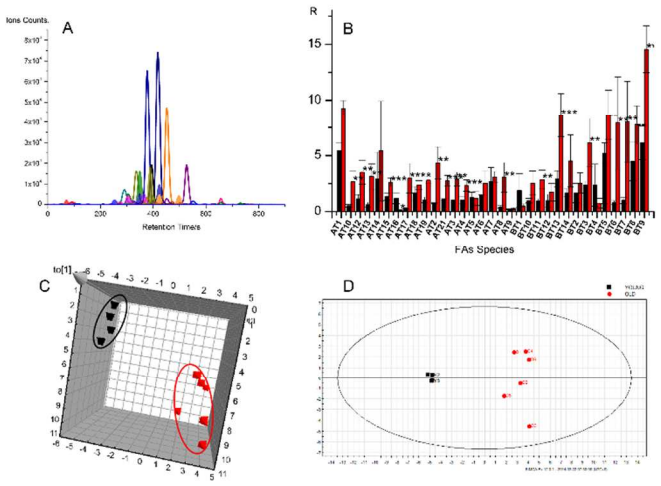


Fig.5 FA compositions in plasma were analyzed by a relative quantification strategy. (A) FAs extracted from plasma were derivatized normally and mixed with their corresponding "heavy" tagged IS. The mixtures were analyzed by HPLC-MRM. Lines refer to plasma samples and patterns refer to the IS. (B) R values calculated from the peak areas of the analytes and the corresponding IS were subjected to OPLS-DA analysis. Several species were determined, as shown in SI.6, because of the significant concentration changes between two groups. The stars indicate the significances (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$; ****: $p < 0.001$). (C/D) On the basis of the OPLS-DA calculations, the two groups of rats (blank: young group n=4, red: old group n=6) were successfully distinguished by either 2 or 3 principle factors.

Conclusions

In this paper, a sensitive, accurate, and full-coverage relative quantification strategy was developed for FA analysis based on a novel isotope derivatization method. Through derivatization, the carboxylic acid groups in the FAs were converted to hydrazides and the analytical sensitivity for the FAs was significantly enhanced. FA quantities as low as 10 fg were detected, which was one thousand times lower compared to the quantities of free FAs detected. Compared with previous FA derivatization methods, the sensitivity was one or two orders of magnitude better. A general MRM condition was developed for analysis of all FA species instead of the molecular specificity MRM optimization; the general MRM condition facilitated FA analysis and guaranteed full coverage. Using corresponding isotopic ISs formed by "heavy" derivatization, we achieved accurate and reliable relative quantification. The new relative quantification strategy has great potential as a powerful tool for biomarker discovery. We applied this strategy to the analysis of biosamples. Several species of FAs in plasma were observed to be capable of distinguishing between young and aged rats and could be considered potential biomarkers for the ageing process.

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Notes and references

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