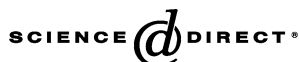


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## CFSUM1 and CFSUM2 in urine from patients with chronic fatigue syndrome are methodological artefacts

Ronald A. Chalmers<sup>\*</sup>, Mark G. Jones, C. Stewart Goodwin, Saira Amjad<sup>1</sup>

*St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK*

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### Abstract

McGregor et al. reported increased levels of an unidentified urinary compound (CFSUM1) in patients with chronic fatigue syndrome (CFS), with reduced excretion of another unidentified compound (CFSUM2), and suggested the possibility of chemical or metabolic 'markers' for CFS. The identity of CFSUM1 as reported was erroneous and the identities of these compounds have remained unknown until now. Urine samples were obtained from 30 patients with ME/CFS, 30 age- and sex-matched healthy controls, 20 control patients with depression and 22 control patients with rheumatoid arthritis. Samples were prepared using the published methods of McGregor et al. to produce heptafluorobutyryl-isobutyl derivatives of urinary metabolites. Alternative preparations utilised isopropyl, *n*-butyl and trifluoroacetyl derivatives. These were separated and identified using gas chromatography–mass spectrometry. CFSUM2 was identified as being partially derivatised [isobutyl ester-mono-heptafluorobutyryl (HFB)] serine. CFSUM1 was identified as partially derivatised pyroglutamic acid, being the isobutyl ester without formation of a HFB derivative. Both CFSUM1 and CFSUM2 are artefacts of the sample preparation procedure and previously reported quantitative abnormalities of CFSUM1 and CFSUM2 in urine from patients with ME/CFS are also artefactual. Pyroglutamic acid may be of primarily dietary origin. The methods used cannot provide reliable qualitative or quantitative data on urinary metabolites. No clinical or biochemical significance can be drawn between these compounds in ME/CFS or any other clinical conditions. © 2005 Elsevier B.V. All rights reserved.

*Keywords:* Urinary metabolites; Gas chromatography–mass spectrometry; Heptafluorobutyryl-isobutyl derivatives

### 1. Introduction

Chronic fatigue syndrome (CFS) [often linked with myalgic encephalomyelitis (ME)] is characterised by severely disabling fatigue, both mental and physical, combined with a variety of other symptoms [1–4] and with a relatively high prevalence in primary care patients [3]. ME/CFS patients describe a variety of infectious and non-infectious antecedents often including a stressful situation and with primary or secondary psychological problems

which may interact in a common pathway [5]. This pathophysiology involves elements of neuroendocrine, neurotransmitter and immune function, all of which individually may be sub-clinical and thus difficult to precisely identify or establish. The high prevalence and debilitating nature of the disorder has provoked extensive scientific and clinical studies in the search for a unifying physiological or metabolic cause. Despite this, the aetiology of the disease remains obscure.

McGregor, Dunstan and colleagues reported increased levels of some urinary compounds in patients with ME/CFS, with reduced excretion in others, suggesting the possibility of one or more chemical or metabolic 'markers' for ME/CFS that may distinguish such patients from those with conditions with overlapping symptomatology [6,7]. One compound observed in significantly increased amounts in comparison to those in control subjects, described as

<sup>\*</sup> Corresponding author. CIMOA, London BioScience Innovation Centre, 2 Royal College Street, London NW1 0NH, UK. Tel.: +44 20 7691 2083; fax: +44 20 7681 9129.

*E-mail address:* [rachalmers@cimoa.org.uk](mailto:rachalmers@cimoa.org.uk) (R.A. Chalmers).

<sup>1</sup> Present address: Davy Faraday Research Laboratory, The Royal Institution, 21 Albemarle Street, London W1S 4BS, UK.

Table 1  
CDC criteria of chronic fatigue syndrome

Impaired memory and concentration
Sore throat
Tender cervical or axillary lymph nodes
Muscle pain
Headaches of a new type or severity
Unrefreshing sleep
Post-exertional malaise for >24 h
Multi-joint pain

“CFSUM1” (chronic fatigue syndrome urinary marker 1), that correlated with core symptom expression in ME/CFS patients [7], was tentatively identified using gas chromatography and mass spectrometry as aminohydroxy-*N*-methylpyrrolidone and was suggested to be a neurotransmitter or pesticide metabolite [6]. Compounds at reduced levels included another unidentified marker, “CFSUM2”, and the amino acids alanine and glutamic acid. Since that time, these workers have continued to evaluate patients with ME/CFS and other associated syndromes including chronic pain in which CFSUM1 in particular has continued to appear correlated with the severity of the disorders. Although CFSUM2 has been tentatively identified as serine (Dr. R.H. Dunstan, personal communication to RAC), this has not been confirmed. Despite the identity of CFSUM1 (and CFSUM2) remaining unknown, McGregor and colleagues have ascribed much significance to this compound in subsequent publications on CFS [8–10] and associated disorders including muscle pain [11] and Irlen Syndrome [12,13] and have also established a commercial base (BioScreen Ltd.; <http://www.bioscreen.com.au/>) for exploitation of metabolic profiling in such patients.

Initial studies by ourselves in patients with ME/CFS and control subjects, using the methods published by McGregor et al. [6], also suggested CFSUM1 was increased in the urine of patients with ME/CFS in comparison to control subjects. However, the identification of this compound as reported by McGregor et al. [6] was questionable since the mass spectral and molecular characteristics published by them and also obtained by ourselves were not in accord with their proposed structure. The original identification of CFSUM1 was confirmed by one of the original authors to be erroneous (Dr. R.H. Dunstan, personal communication to RAC, 1997) although this has not been acknowledged in subsequent publications. The present work was designed to unequivocally identify and characterise CFSUM1 and CFSUM2, as a basis for a better understanding of these putative chemical or metabolic markers for CFS and their relationship to the underlying aetiology of the disease.

## 2. Patients and materials

These studies were approved by the Wandsworth Local Research Ethics Committee and informed consent was

obtained from all participants before involvement in the studies.

Patients with ME/CFS were defined according to the International Oxford and CDC criteria. The CDC criteria are shown in Table 1: patients with four or more of these symptoms were judged as meeting the criteria for CFS. None of the patients showed symptoms of depression. A total of 30 patients were recruited, 12 male and 18 female with an overall age range of 26–84 years (Table 2).

Twenty patients with depression but without CFS and 22 patients with rheumatoid arthritis as a representative inflammatory disease were recruited as control patients (Table 2). All were free from concomitant diseases of liver, kidney and heart and were not receiving medications that would interfere with the analyses being undertaken.

Thirty healthy subjects were also recruited as a further control group, matched as closely as possible to age and sex distribution of the patients with CFS (Table 2) and also with their general lifestyle. Subjects on medications and who were smokers were excluded.

All patients and control subjects were provided with a questionnaire, a shortened form of that of Ray et al. for ME/CFS patients [14], to assess (A) their somatic symptoms and cognitive difficulties and (B) disability and recent course of illness. The scores for each section were combined to provide a Sickness Impact Profile Score (SIPS). The average SIPS for patients with ME/CFS was 65, compared to 13 for the age- and sex-matched healthy controls, 30 for the patients with depression and 46 for patients with rheumatoid arthritis (Table 2). The scores for the latter two groups of control patients were less reliable since incomplete questionnaires were returned from several patients.

Patients were asked to provide two samples of urine, the first sample passed in the morning for initial analysis using the method of McGregor et al. [6] and a timed 6-h

Table 2  
Patient and control groups

Patient group	Number (M= male; F= female)	Age range (median)	Sickness impact profile score <sup>a</sup> (median and range)
ME/CFS	12 M 18 F	26–63 (44) 26–84 (45)	65 (35–98)
Healthy	12 M 18 F	20–66 (45) 26–79 (45)	12 (0–34)
Depression	8 M 12 F	34–71 (48) 24–59 (46)	[30 (6–51)] <sup>b</sup>
Rheumatoid arthritis	5 M 17 F	43–67 (57) 39–65 (53)	[46 (22–85)] <sup>b</sup>

<sup>a</sup> Based upon a questionnaire [14] to assess (A) their somatic symptoms and cognitive difficulties and (B) disability and recent course of illness. The scores for each section were combined to provide the Sickness Impact Profile Score (SIPS).

<sup>b</sup> The scores for the latter two groups of control patients were less reliable since incomplete questionnaires were returned from several patients.

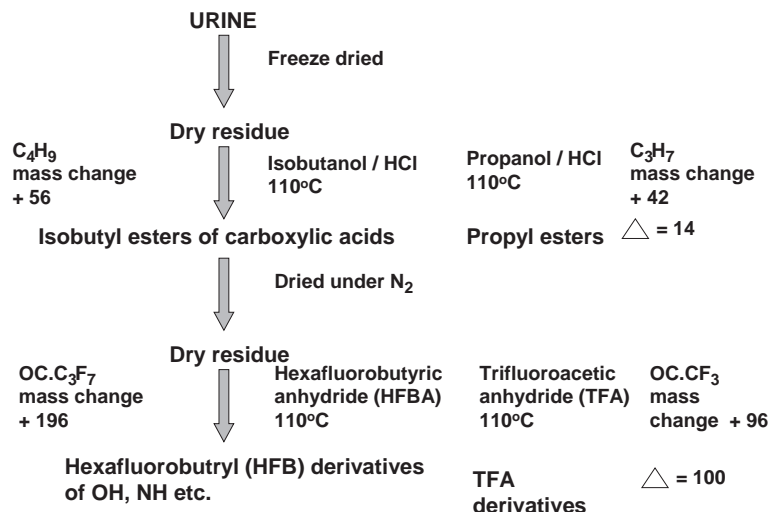


Fig. 1. General schematic of sample preparation and derivatisation methods used, illustrating the mass increments obtained using the various derivatisation reagents.

fasting collection for quantitative metabolite analysis. First morning samples were initially analysed immediately [6] but further study showed no differences after freezing and other samples were stored deep frozen at  $-20\text{ }^\circ\text{C}$  until analysed.

### 3. Methods

The procedure followed that of McGregor et al. [6] as closely as possible. Urine was centrifuged and 200  $\mu\text{l}$  of the supernatant was freeze-dried until completely dry. The freeze-dried residue was treated with dry 200  $\mu\text{l}$  iso-

butanol/HCl and heated at  $110\text{ }^\circ\text{C}$  for 1 h to form the isobutyl esters of any carboxylic acids present in the residue. The mixture was then reduced to dryness under a stream of dry nitrogen and the dry residue treated with 50  $\mu\text{l}$  ethyl acetate and 20  $\mu\text{l}$  heptafluorobutyric anhydride (HFBA) at  $110\text{ }^\circ\text{C}$  for 30 min to form the heptafluorobutryl derivatives of hydroxy, imino and amino groups in any compounds present. After cooling, the sample was again reduced to dryness under a stream of dry nitrogen and freeze-dried for 5 min. The dry residue was redissolved in 200  $\mu\text{l}$  of ethyl acetate for gas chromatography (GC) and mass spectrometry (MS). It was found most important to maintain a completely anhydrous

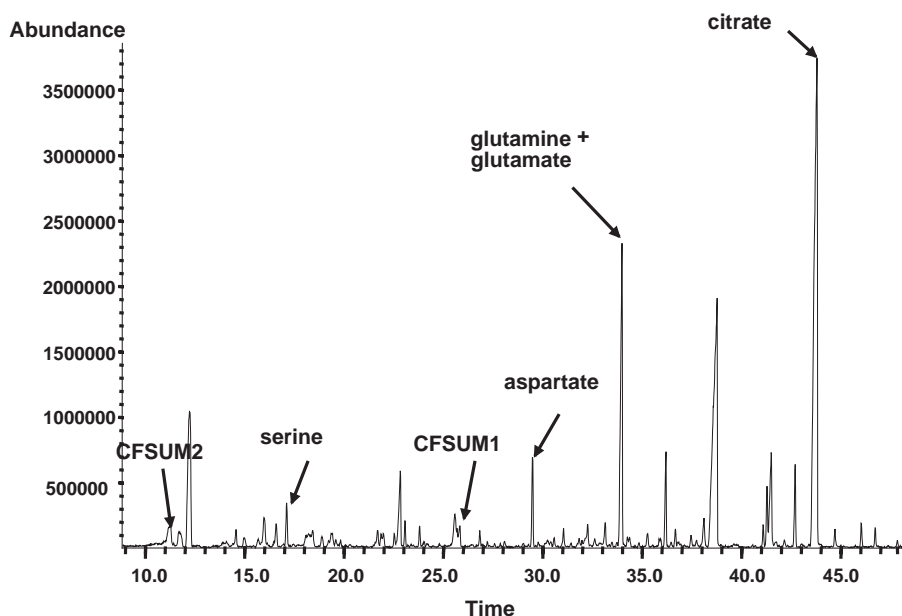


Fig. 2. Typical total ion chromatogram obtained from a sample of urine extracted and derivatised as described in the text using isobutanol/HCl and HFBA. The relative positions of CFSUM1, CFSUM2 and some major components are illustrated.

environment throughout these procedures to avoid hydrolysis of the prepared HFB derivatives.

The derivatised compounds were separated by programmed temperature capillary gas chromatography on CP-Sil5 columns in a Hewlett-Packard 5890 gas chromatograph coupled to a HP 5970 mass selective detector in order to obtain the electron impact (EI) mass spectra of the eluting compounds. In alternative analyses, chromatography was carried out in a similar manner using a HP 5980 Series II gas chromatograph coupled to a HP5972 mass selective detector using chemical ionisation with methane as reagent gas.

The procedure was varied in different experiments with dry isopropanol/HCL or *n*-butanol/HCL replacing isobutanol/HCL and with trifluoroacetic anhydride (TFA) replacing HFBA in order to effect different mass changes on derivatisation as an aid to identification of the compounds present. A general schematic is shown in Fig. 1 illustrating the mass increments obtained using the different derivatisation methods.

## 4. Results and discussion

### 4.1. Preliminary structural analysis of CFSUM1

Fig. 2 shows a typical total ion chromatogram of a sample from a patient with ME/CFS obtained following derivatisation with isobutanol/HCl with the eluting positions of CFSUM1 and CFSUM2 and some major components. The chromatogram is comparable to that shown by McGregor et al. [6] and other components were eluted with similar retention times. Identifications of major components were made by comparison to library spectra where available and by reference to spectra obtained using authentic standards taken through the same derivatisation procedures.

Fig. 3a shows the EI mass spectrum of CFSUM1 with a  $M^+$  ion at  $m/z$  185, a major fragment ion at  $m/z$  84 and other ions at  $m/z$  169, 69, 57 and 41. This spectrum is closely similar to that shown by McGregor et al. [6] and confirmed the same compound that was found in the present work, although there was no ion observed at  $m/z$

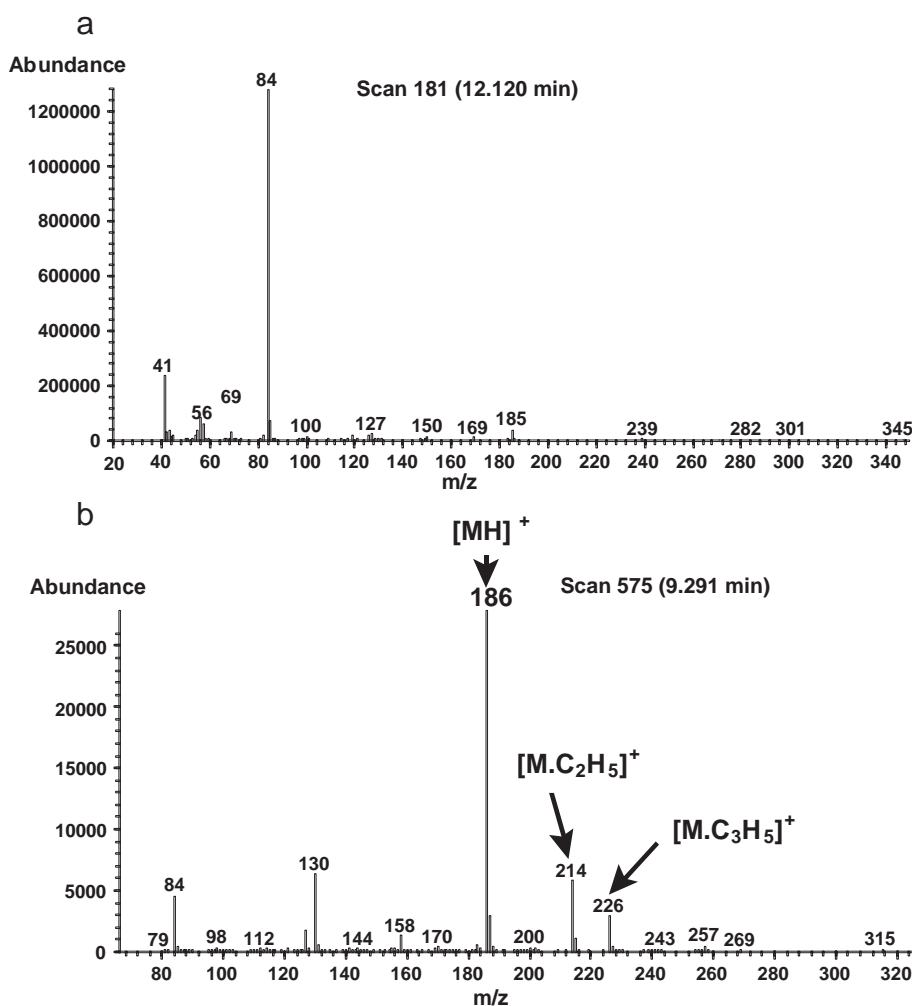


Fig. 3. (a) Electron impact (EI) mass spectrum of the isobutyl-HFB derivative of CFSUM1. Note  $M^+$  at  $m/z$  185 with the major fragment ion at  $m/z$  84 and with absence of an ion at  $m/z$  354. (b) Chemical ionisation (CI) mass spectrum of CFSUM1 obtained using methane as reagent gas. Confirming the molecular mass of CFSUM1 to be 185.

354. Fig. 3b shows the CI mass spectrum of CFSUM1, confirming the molecular mass of (HFB-isobutyl) CFSUM1 to be 185.

Fig. 4 shows the EI mass spectra of the HFB-isobutyl derivative of CFSUM1 (as also shown in Fig. 3a) and the EI mass spectrum of the HFB-isopropyl derivative of CFSUM1. These clearly show the mass change that occurs with use of isopropanol/HCL reagent and confirms the presence of a single carboxylic acid group on the parent molecule. Fig. 5 shows the EI mass spectra of the HFB-isobutyl derivative of CFSUM1 (as also shown in Fig. 3a) and of the TFA-*n*-butyl derivative of CFSUM1, indicating there is no group present on CFSUM1 that can react with either HFBA or TFA under the conditions used. Thus CFSUM1 reacts solely with the alcoholic reagent (isobutanol, isopropanol or *n*-butanol) in the derivatisation process. Allowing for the mass increment produced by the alcohol moiety, these results show that the molecular mass of CFSUM1 is 129. From the EI spectra (including the prominent  $m/z$  84 fragment) and molecular mass, CFSUM1 would be likely to have a nitrogen-containing ring structure and, from the above results, a single carboxylic acid group; this would give an empirical formula of  $C_6H_{11}O_2N$  or  $C_5H_6O_3N$ . The possible structures with a molecular mass of 129 are shown in Fig. 6.

#### 4.2. Structural analysis of CFSUM2

Fig. 7 shows the EI mass spectrum of the diHFB-isobutyl derivative of the peak labelled as serine in Fig. 2, confirmed as serine by comparison to an authentic standard derivatised in the same manner. HFB-isobutyl derivatives of amino acids are characterised by ‘molecular’ ion at  $M-101$ , in diHFB-isobutyl serine at  $m/z$  452. Serine forms a mono-isobutyl ester on its carboxylic acid group and has both a hydroxyl group and an amino group on which the hydrogens may be substituted by HFB (Fig. 8) to give a derivative with molecular mass 553 and the spectrum shown in Fig. 7. The hydrogen on the hydroxyl group is more easily replaced than those on the amino group and thus a mono-HFB derivative may be formed initially in the derivatisation process, with a molecular mass of 357 (Fig. 8). The EI mass spectrum of isobutyl-mono-HFB serine is shown in Fig. 9 together with the EI mass spectrum of the peak labelled as CFSUM2 in Fig. 2. The spectra are virtually identical and confirm the identity of CFSUM2 as partially derivatised serine. This confirms the original tentative origin of this compound (Dr. R.H. Dunstan). However, since under the preparation conditions used the amino acid gives two derivatives (the

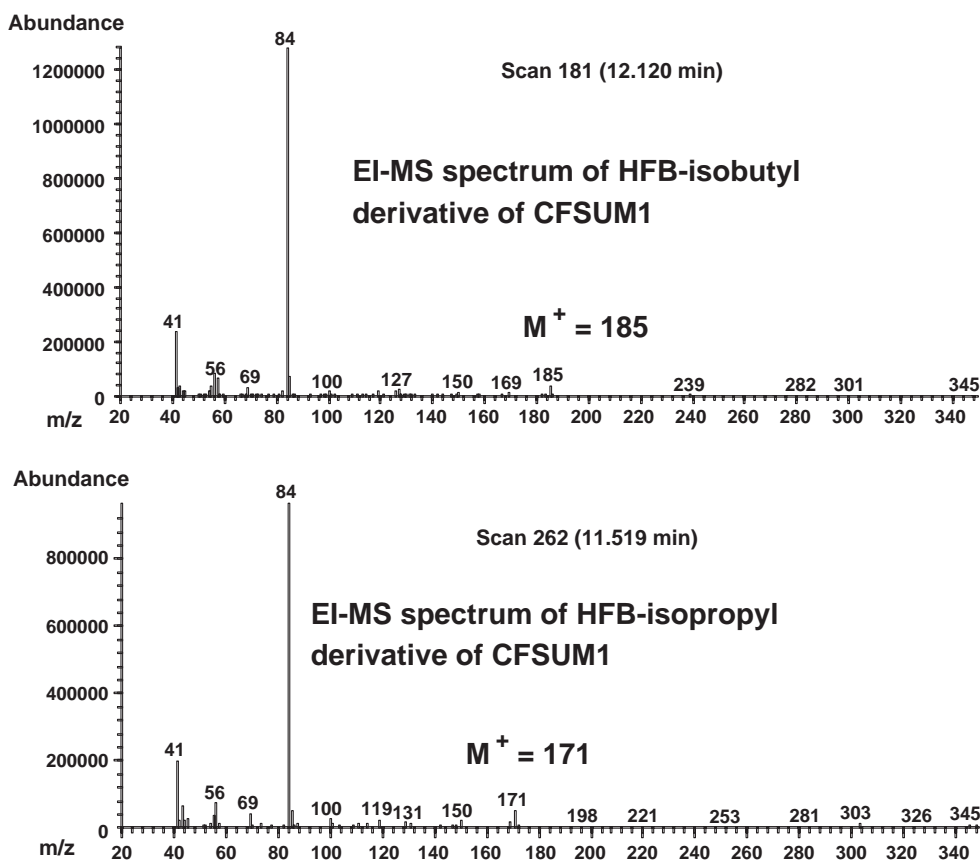


Fig. 4. Electron impact (EI) mass spectra of CFSUM1 as the HFB-isobutyl derivative (upper) and as the HFB-isopropyl derivative (lower). Molecular ion mass change of  $-14 m/z$  confirming the presence of a single carboxylic acid group.

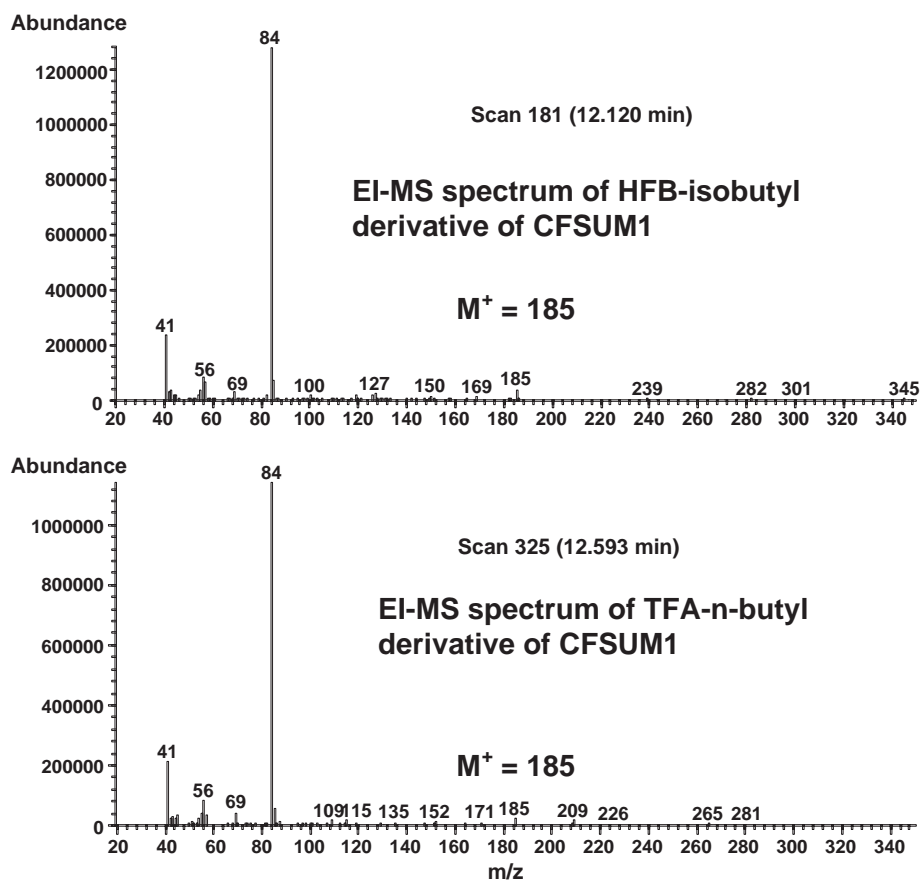


Fig. 5. The electron impact (EI) mass spectra of CFSUM1 as the HFB-isobutyl derivative (upper) and as the TFA-*n*-butyl derivative (lower). Showing no mass change with use of the different reagents.

mono-HFB isobutyl and the di-HFB isobutyl derivatives) in varying amounts, serine cannot be accurately quantified using these methods. Derivatisation of phosphoserine also produces a peak due to CFSUM2 (Dr. R.H. Dunstan, personal communication to RAC), indicating phosphoserine is degraded to serine under the preparation conditions used. The degradation of more complex molecules suggests other presently unidentified compounds produced by the preparative process used by McGregor et al. [6], for example UM15, etc., may also arise through artefactual processes.

#### 4.3. Complete structural analysis of CFSUM1

Observation of the occurrence of partial derivatisation of serine in the complex urinary matrix obtained using the method of McGregor et al. [6] suggested partial derivatisation of other compounds present in the matrix also occurs and may lead to some of the other 'urinary markers' that remain unidentified in their procedure, including CFSUM1. One of the possible structures for CFSUM1 shown in Fig. 6 is pyroglutamic acid, a common constituent of urine. Pyroglutamic acid contains a single carboxylic acid group and also a ring imino group with a potentially replaceable hydrogen. Thus pyroglutamic acid

in derivatisation with isobutanol/HCl and HFBA will initially form an isobutyl ester with a molecular mass of 185 and subsequently a mono-HFB derivative with a molecular mass of 381 (Fig. 10). Fig. 11 shows the EI mass spectra of authentic isobutyl pyroglutamic acid and of HFB-isobutyl pyroglutamic acid (note  $M-101=280$ ). The spectrum of the simple isobutyl ester is identical to that of CFSUM1 (Fig. 12), confirming CFSUM1 to be pyroglutamic acid, partially derivatised in the procedure of McGregor et al. [6].

#### 4.4. Occurrence and origins of pyroglutamate in human urine

As the name implies, pyroglutamic acid may be formed from glutamic acid by heating (under hydrous conditions) and occurs in heat-treated foods from which it can be absorbed and excreted into the urine unchanged. Pyroglutamic acid itself may also be an artefact if heat is used in the preparation of the urine extract or in the derivatisation process. The procedure of McGregor et al. [6] involves heating the sample under anhydrous conditions at 110 °C at two stages of the preparation process and we have shown that under these conditions pyroglutamate may also interconvert to glutamate (data not shown). Pyroglutamic

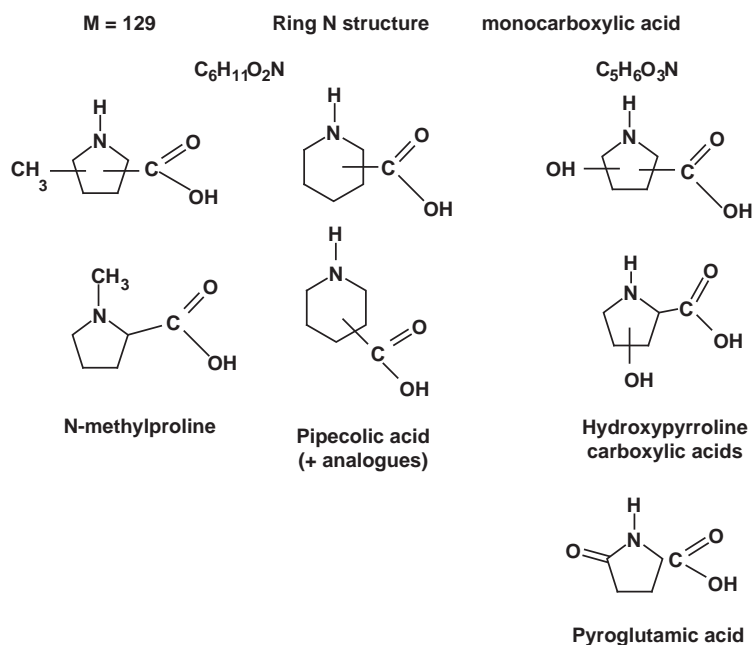


Fig. 6. Possible structures for CFSUM1. Assuming a nitrogen-containing ring structure with a single carboxylic acid group present and a molecular mass of 129.

acid (pyrrolidone carboxylic acid; 5-oxo-proline) is a common constituent of human urine, being derived both from heat-treated and other foodstuffs and endogenously from the gamma-glutamyl cycle [15]. With a primarily

dietary origin in human subjects who do not have the rare inherited metabolic disorder pyroglutamic aciduria, the metabolite does not have any clinical or metabolic significance.

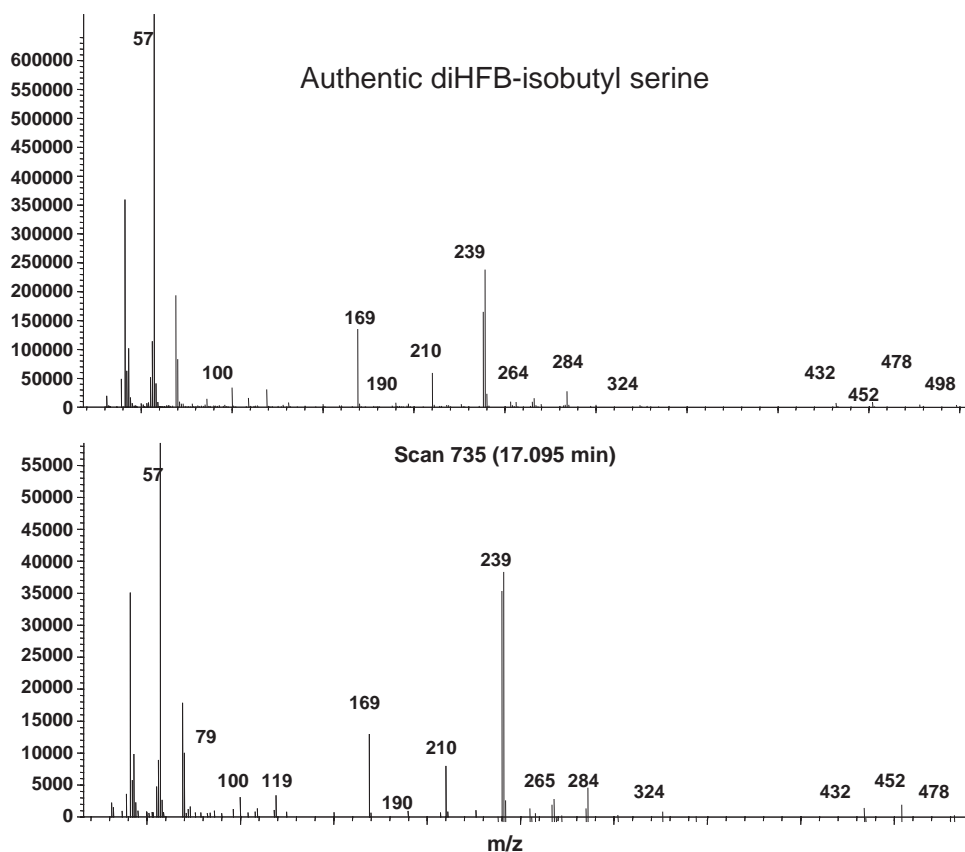


Fig. 7. EI mass spectrum of the peak labelled as serine in Fig. 1 (lower) compared to authentic diHFB-isobutyl serine.

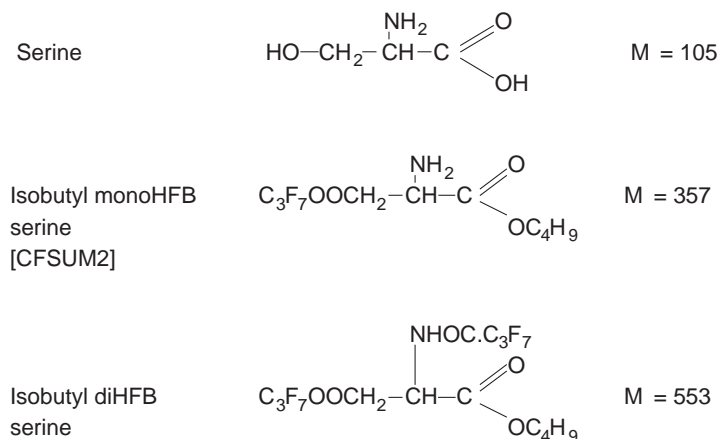


Fig. 8. Serine and derivatisation with isobutanol/HCL and HFBA, showing the structures of the di-HFB and mono-HFB derivatives.

#### 4.5. Quantification of CFSUM1 and CFSUM2 using the method of McGregor et al. [6]

Table 3 shows the relative amounts of CFSUM2 and serine and of CFSUM1 and glutamate, determined and quantified in urine according to the method of McGregor et al. [6] and expressed as percentages of the total peak area in the total ion chromatogram. Approximately equivalent amounts of CFSUM2 and serine are observed

whereas the amount of CFSUM1 is relatively small in comparison to that of glutamic acid. The occurrence of two derivatives of serine, with quantification of only one, may explain the apparent reduced excretion of CFSUM2 (and serine) in patients with ME/CFS observed by McGregor et al. [6,7]. Glutamate is present in comparatively large amounts and itself may be the product of glutamine due to the high degree of thermal lability of glutamine. Thus relatively low fluctuations in the degree of interconversion

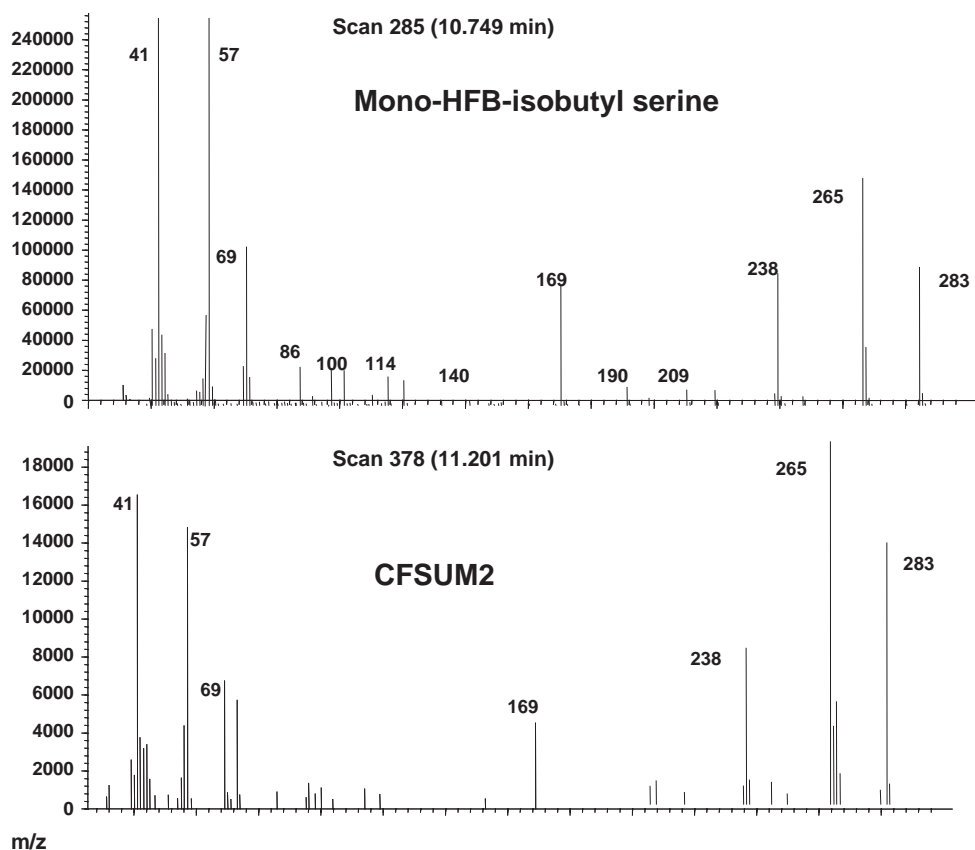


Fig. 9. EI mass spectra of authentic mono-HFB-isobutyl serine (upper) and the peak labelled as CFSUM2 in Fig. 2 (lower).

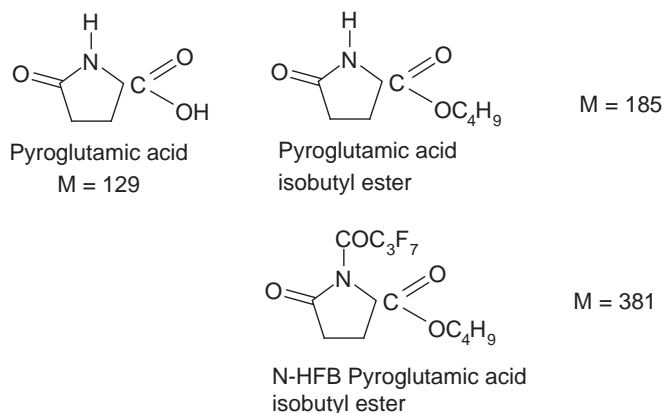


Fig. 10. Pyroglutamic acid and its isobutyl ester and *N*-HFB-isobutyl derivative.

of glutamate with pyroglutamate may produce major variations in the apparent amounts of the latter compound (“CFSUM1”) that are, however, biochemically and clinically irrelevant since they are the product of an artefactual process. Additionally, as indicated above, the majority of the pyroglutamate observed in human urine is of dietary origin and thus not of biochemical or clinical relevance. Values are given for the four study groups, patients with ME/CFS, healthy controls, patients with depression and patients with rheumatoid arthritis, obtained under as near identical conditions of preparation as possible; no significant differences were observed between the values obtained for the four groups of subjects, in particular

between patients with ME/CFS and age-, sex- and life-style-matched healthy control subjects.

Urinary data expressed using the method of McGregor et al. [6] may be subject to the degree of dilution of the urine samples from individual subjects. Possible differences between the groups were examined by determination, using a modified Jaffé method, of the urinary creatinine concentrations as a measure of urinary dilution. Concentrations (expressed as mmol/L, mean  $\pm$  standard deviation) in the groups studied were: healthy controls  $7.38 \pm 5.22$ , CFS patients  $9.52 \pm 5.24$ , patients with depression  $8.21 \pm 4.99$ , patients with rheumatoid arthritis  $7.79 \pm 5.46$ . Statistical analysis using unpaired *t*-test, Mann–Whitney *U*-test and

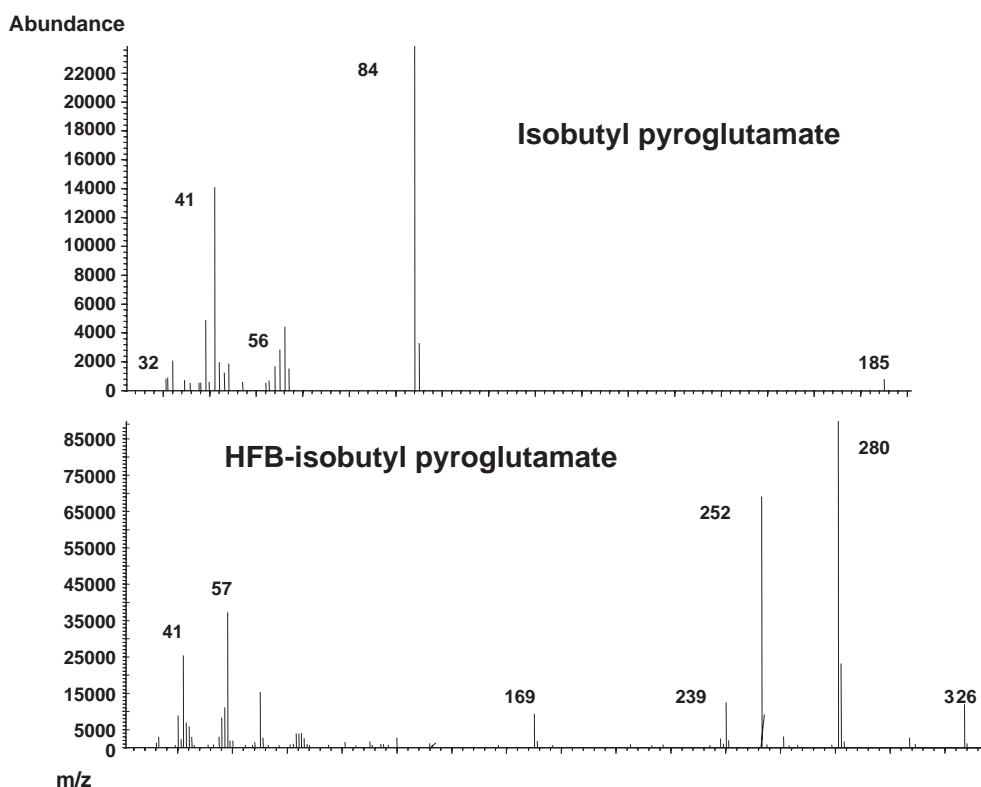


Fig. 11. Electron impact (EI) mass spectra of isobutyl pyroglutamic acid (upper) and *N*-HFB-isobutyl pyroglutamic acid.

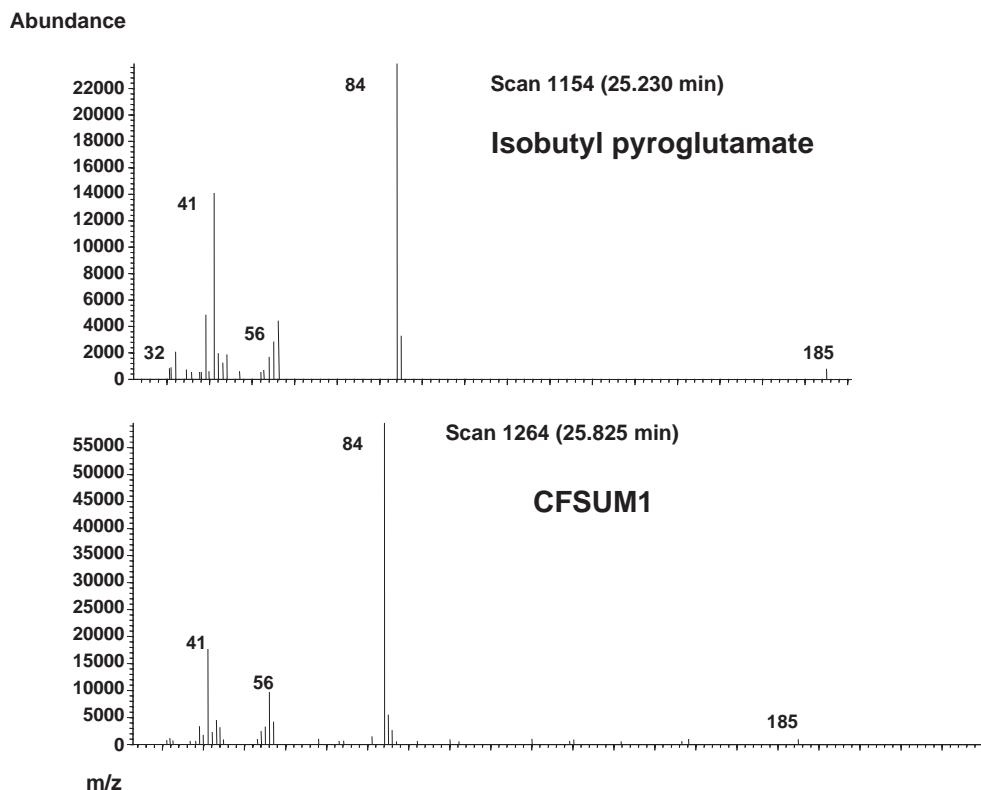


Fig. 12. Electron impact (EI) mass spectra of isobutyl proglumatic acid and CFSUM1 from the urine of a patient with ME/CFS.

one-way ANOVA showed no significant differences between any of the groups studied.

## 5. Conclusions

Formation of isobutyl-HFB derivatives at 110 °C in complex matrices of freeze-dried urine may be incomplete. This results in partial derivatisation and artefact formation. CFSUM2 is derived from serine and is incompletely derivatised mono-HFB-isobutyl serine. CFSUM1 is derived from pyroglutamic acid and is partially derivatised, being the isobutyl ester, with no formation of a HFB derivative. Both CFSUM1 and CFSUM2 are artefacts of the sample preparation procedure developed by McGregor et al. [6].

Table 3

Urinary metabolites in patients with ME/CFS and control groups [healthy subjects, patients with depression and patients with rheumatoid arthritis], determined according to the method of McGregor et al. [6]

Patient group	ME/CFS	Healthy	Depressed	Rheumatoid arthritis
Number in group	30	30	20	22
CFSUM2	1.34±0.67	1.34±0.76	1.05±0.51	1.38±0.61
Serine	1.44±0.56	1.52±0.63	1.88±0.50	1.80±1.10
CFSUM1	1.70±1.55	1.14±0.83	1.06±0.59	1.86±1.56
Glutamate	9.58±3.18	9.54±4.04	9.68±3.54	9.72±3.76

Expressed as percentage of total peak area of the total ion chromatogram (mean±standard deviation).

The procedure used may also explain the nature of other unidentified “urinary markers” (UM15b, etc.) that are referred to in papers by McGregor, Dunstan and colleagues [6–13] and may also be the result of artefactual and partial derivatisation processes. Thus the analytical procedure described by McGregor et al. [6] cannot be used to provide reliable qualitative or quantitative data on urinary metabolites, including amino acids and organic acids. The latter metabolites should be determined using carefully validated and conventional methods to obtain meaningful data. Nevertheless, in the present work, comparison between the amounts of CFSUM1 and CFSUM2 measured in the urine of patients with ME/CFS and healthy control subjects under carefully controlled conditions to produce comparable results showed no differences, suggesting previously reported differences [7–9] were also artefactual in origin. Most importantly, since CFSUM1 and CFSUM2 are artefacts formed in variable amounts depending upon the exact conditions used in sample preparation and since the precursor of CFSUM1 (pyroglutamate) may be primarily of dietary origin, no significance can be drawn between these compounds in ME/CFS or any other clinical conditions.

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