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**Amino acid composition, including key derivatives of eccrine sweat,  
potential biomarkers of certain atopic skin conditions**

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**Synopsis**

The free amino acid (AA) composition of eccrine sweat is different from other biological fluids, for reasons which are not properly understood. We undertook the

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detailed analysis of the AA composition of freshly isolated pure human eccrine sweat, including some of the key derivatives of AA metabolism, to better understand the key biological mechanisms governing its composition. Eccrine sweat was collected from the axillae of 12 healthy subjects immediately upon formation. Free AA analysis was performed using an automatic AA analyzer after ninhydrin derivatisation. Pyrrolidine-5-carboxylic acid (PCA) and urocanic acid (UCA) levels were determined using GC/MS. The free AA composition of sweat was dominated by the presence of serine accounting for just over one fifth of the total free AA composition. Glycine was the next most abundant followed by PCA, alanine, citrulline and threonine, respectively. The data obtained indicates that the AA content of sweat bears a remarkable similarity to the AA composition of the epidermal protein profilaggrin. This protein is the key source of free AA's and their derivatives that form a major part of the natural moisturising factor within the stratum corneum (SC), and plays a major role in maintaining the barrier integrity of human skin. Since perturbations in the production of NMF can lead to abnormal barrier function and can arise as a consequence of filaggrin genotype, we propose the quantification of AA's in sweat may serve as a non-invasive diagnostic biomarker for certain atopic skin conditions i.e. atopic dermatitis (AD).

**Keywords:** Filaggrin - natural moisturizing factor - serine - stratum corneum

## Introduction

Eccrine sweat is a dilute electrolyte solution whose primary function is to control body temperature via evaporative cooling [1]. Although sweat is primarily composed of water, previous studies have shown that a diverse array of organic compounds are

also present [2,3]. The presence of free amino acids (AA) in sweat has been known since 1910 when serine was isolated from sweat as  $\beta$ -naphthalinesulfoserine [4]. Subsequent studies have described the concentration of free AA's in eccrine sweat, which were collected under various environmental conditions [5,6,7,8], with the reported levels varying significantly. One reason for these large differences is the diverse range of sampling methods used to obtain the sweat, rather than actual differences in AA concentrations; with the published data in some instances being more an equilibrium measure of the AA concentration present at the surface of the stratum corneum (SC), rather than that present in the secreted sweat [9]. In contrast, the AA profiles of sweat obtained in the different studies exhibit a constant qualitative pattern. However, this profile does not reflect that of other biological fluids, or match that of the average AA composition across the human proteome [10].

The human epidermis serves three important functions in providing a barrier against the (i) transcutaneous loss of water, (ii) infection by microbial pathogens, and (iii) penetration of xenobiotics [11]. The outer most layer of the epidermis, the SC, is the major component providing this protective function. The SC is comprised of anucleate corneocytes, which are surrounded by a cornified cell envelope, composed of structural proteins including involucrin, loricrin and trichohyalin. These proteins are cross-linked to proteins of neighbouring corneocytes through the action of several transglutaminases to form a rigid, mechanically resistant structure [12]. A complex mixture of lipids (ceramides, cholesterol and free fatty acids) form intercellular lamellae around the corneocytes to help form a complete barrier [13]. The corneocytes themselves contain keratin intermediate filaments embedded within a transient filaggrin matrix. Filaggrin is deiminated and ultimately completely degraded to release its component AA's, a mixture of hygroscopic AA's, termed

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“natural moisturizing factor” (NMF) [14]. NMF contribute to epidermal hydration and barrier function [15]. Perturbations in the levels of NMF’s in the SC are associated with general skin conditions such as winter and senile xerosis and with certain skin diseases e.g. ichthyosis vulgaris and atopic dermatitis (AD) [10]. In the present study we analysed the free AA content of pure eccrine sweat, including some of the key derivatives of AA metabolism, under conditions designed to keep to an absolute minimum the equilibration with and contamination from skin surface components; to better understand the key biological mechanisms governing its composition, which to date have not been thoroughly investigated.

## **Materials and methods**

### **Subjects**

This study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. The local ethics committee approved the protocol before commencement of the study and all subjects gave written informed consent. Subjects were aged between 20 to 41 years old (mean age 26 years old) and in good general health (BMI 19 to 29). A total of 12 healthy subjects (6 males and 6 females) participated in the study.

### **Sweat collection**

Subjects were requested to refrain from using antiperspirant products in the 3 week pre-test period (i.e. they could use deodorant formulations). Subjects were also required to shave their underarms 48 hrs prior to the sweat collection. Following shaving no underarm product was used until after the test period, although washing

with un-fragranced soap was permitted. Subjects abstained from using any underarm products 48 hrs prior to testing. Subjects refrained from drinking alcohol for 24 hrs prior to providing a sweat sample and were asked not to drink caffeinated products on the morning of the study. Subjects were instructed to drink 500 ml of a non-alcoholic liquid prior to going to bed the night before the sweat collection. On the morning of the collection subjects were asked to eat and drink as normal apart from restricting their caffeine intake.

On the test day subjects were required to rinse and dry their axilla with water then enter the hot room. Sweat collection involved subjects being seated in an environmental chamber at 40 °C: 60 % relative humidity. Subjects initially underwent a warm up period of between 15 and 40 min (subject to individual response). Their underarm area was then wiped and as the subjects continued to sweat from their axillae, the sweat droplets which formed were promptly collected using a Gilson positive displacement pipette and placed directly in a “Low Binding” 1.5 ml Eppendorf tube, which was kept at 4 °C. The collection period lasted for approximately 20 min. Once sweat collection was complete the samples were stored at -70 °C.

To determine sweat rates, subjects were required to rinse and dry their axilla with distilled water then enter the hot room. Environmental conditions in the hot room were set at 40.0 °C and 40.0 % relative humidity (RH). Subjects initially underwent a warm up period of 40 minutes. After the warm-up period the axilla was rinsed clean again and blotted dry with a disposable paper towel. Sampling was performed from both the left and right axillae. The collection period lasted for 20 minutes. Sweat rate was calculated gravimetrically from the amount of sweat collected on pre-weighed absorbent gauze pads held in each underarm for the 20 minutes sweat collection

period. After the 20 minutes sweat collection period the pads were re-weighed to determine the total amount of sweat secreted during the sampling period.

### **Amino acid analysis**

Free amino acid analysis was performed at Alta Bioscience, University of Birmingham, UK; by cation chromatography on a Biotronic LC 5001 automated amino acid analyser, according to a modified method of Spackman *et al* [16]. Quantification of pyrrolidine-5-carboxylic acid (PCA) and urocanic acid (UCA) were performed using gas chromatography-time of flight-mass spectrometry (GC/MS). Samples were dried (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap; Thermo Life Sciences, Basingstoke, UK) and stored at 4 °C until analysis. A series of calibration standard solutions were prepared in water and contained both PCA (100-2000 µmol/l) and UCA (5-500 µmol/l). Sweat samples and standard solutions were chemically derivatised applying a two-step process (oximation and trimethylsilylation) as described previously [17]. GC/MS analysis was performed as previously described [18] applying a LECO Pegasus III electron impact mass spectrometer (LECO, Stockport, UK) coupled to an Agilent 6890 gas chromatograph (Agilent Technologies, Cheshire, UK). Standard calibration curves were constructed with data acquired at the start of the analytical run followed by analysis of the sweat samples. Comparison of the peak area data between the calibration curves and samples allowed the calculation of the PCA and UCA concentrations. For each of the analytical procedures employed 500 µl of sweat was required.

## Statistical analysis

Data are presented in the form of mean and standard deviation. A Students paired  $t$  test was used to compare values obtained between the male and female subjects.  $P$  values  $< 0.05$  are considered statistically significant.

## Results and discussion

### Amino acid analysis

Sweat was collected immediately upon visible droplet formation on the surface of axillary skin. This was to minimise as much as possible contamination of the sweat from water soluble SC surface compounds. Ultimately, some equilibration of the sweat with water soluble factors readily extractable from the SC surface would have occurred prior to sampling. However, short of cannulating individual sweat glands at the skin surface, this technique provides the most reliable method for attempting to collect uncontaminated sweat. This is the unique aspect of this study compared with previous studies which claim to analyse the AA content of eccrine sweat. These other studies collected sweat from the skin surface using a wide range of sampling techniques including rollers, pouches and blankets, all of which will have resulted in significant equilibration of the collected sweat with sweat soluble compounds either present on the skin surface or readily extracted from the superficial layers of the SC [5,6,7,8].

The free AA concentration of eccrine sweat obtained from 6 healthy men and 6 healthy women are provided in Table 1. Using the analytical procedures employed taurine, hydroxyproline and cysteine could not be detected in any of the samples.

Women tended to have higher concentrations of free AA's in their sweat than men, although these differences were non-significant. Women also exhibited reduced axillary sweat rates compared to men, but again these differences were non-significant. However, the fact that the concentration of AA in female sweat was higher compared to males may be a function of sweat rate but there is insufficient data to fully support this proposition. Inter-subject variations in the concentration of free AA's were relatively large. Previous studies describing the concentration of free AA's in human sweat differ greatly [5,6,7,8], but the values quoted are generally much higher than those presented in the current study. Much of this discrepancy is probably due to the disparate methods used for sweat induction, sampling, and analysis. Therefore, it is impracticable to directly compare the absolute concentrations of free AA's obtained in this study with those reported previously.

The profiles of the free AA's in the sweat collected from the axillae of both men and women, showed a consistent qualitative pattern. There is considerable consistency in the literature regarding the relative abundances (RA) of the free AA's present in sweat. The consensus finding of previous studies observed that serine, glycine, and alanine are the most abundant AA's detected. The data obtained in the present study concurs with this finding. Serine was by far the most abundant AA present in the sweat samples, accounting for just over 20% of the total AA composition. Glycine was the next most abundant AA representing approximately 15 % of the total composition. The RA of alanine, citrulline, threonine and glutamic acid constituted the next most abundant AA's, respectively.

Free AA's including derivatives such as PCA and UCA form a major component of NMF in the SC (~ 50%) [14]. These AA's are primarily derived from filaggrin breakdown during the latter stages of epidermal differentiation [19,20].

Filaggrin is synthesized as a heavily phosphorylated precursor protein (profilaggrin) that consists of 10-12 filaggrin repeats [21]. During keratinocyte terminal differentiation profilaggrin is dephosphorylated and undergoes limited proteolysis to yield mature filaggrin monomers. Filaggrin is then further modified by the peptidyl arginine deiminase family of enzymes prior to the complete hydrolysis of the protein into free AA's and their derivatives within the SC. These AA's form the major proportion of the cutaneous, NMF [14]. The presence of relatively high levels of citrulline in the SC reflects the activity of the peptidyl arginine deiminase enzyme that converts peptide bound arginine into peptide bound citrulline within filaggrin, which is subsequently liberated during protein hydrolysis.

Some of the filaggrin derived AA's undergo further transformations to produce AA derivatives which play key roles in skin homeostasis. Pyrrolidone-5-carboxylic acid (PCA) is produced from glutamine, it is a major constituent of NMF and is one of its most hygroscopic components [22]. Histidine is converted to trans-urocanic acid (t-UCA) by the catalytic action of histidase [19,23]. Subsequent isomerisation of t-UCA via UV irradiation yields cis-UCA. These derivatives serve as major chromophores and in the case of cis-UCA as a modulator of immune function in skin [24]. The concentrations of PCA and UCA present in pure eccrine sweat as analysed by GC/MS are displayed in figure 1. The concentration of PCA can be seen to be particularly high in comparison to the majority of AA's found in sweat, with UCA also detected at appreciable levels.

## **Relative abundance of amino acids detected in sweat compared to the amino acid composition of skin derived proteins**

When the concentrations of PCA and UCA are included in the total sweat AA composition, and the RA of the different AA's are ranked, an interesting pattern begins to emerge (Fig. 2). Serine is still the most abundant AA, glycine is next followed by glutamine and its derivative PCA, then arginine and its derivatives citrulline and ornithine. The RA of the top ten AA's in sweat is almost identical to that of filaggrin, but different to that of other major epidermal component proteins i.e. keratins, loricrin and involucrin. Although keratin I and loricrin exhibit some similarities, there are still major discrepancies in their AA composition when compared to sweat and filaggrin. For example, leucine and cysteine are major components of keratin I and loricrin, respectively, but form only minor components of the AA composition of sweat and filaggrin. Other amino acids commonplace in mammalian proteins are essentially absent from filaggrin and are also conspicuous by their absence from sweat e.g. methionine, cysteine and tryptophan.

The results provide strong evidence that the AA content of the SC strongly influences the AA content of sweat, with the hydrolysis of filaggrin in the SC being a key mechanism determining this relationship. This finding suggests that the conduit of sweat along the duct results in its equilibration with the available free AA's in the surrounding tissue, causing the AA composition of the sweat secreted immediately onto the skin surface to be very similar to that of the SC. The similarities of the RA of the AA composition of sweat and filaggrin are extremely compelling. Particularly considering the relatively low levels of lysine and leucine present in sweat and filaggrin, which by comparison comprise a marked portion of the AA composition of the other major epidermal proteins, plasma and interstitial fluid [25,26]. Previous

work has also demonstrated the presence of keratohyalin granules, which contain profilaggrin, within the mid and upper regions of the acrosyringium of eccrine sweat glands [27]. This observation is entirely consistent with the proposal that there is considerable equilibration of filaggrin derived AA's into eccrine sweat directly from the ductal lining, during the passage of sweat to the skin surface. Whereas it has been recognised that eccrine sweat is a likely source of other non-free AA major contributors of the NMF e.g. lactate and urea [28,29], it has not previously been realised that free AA levels measured in the surface layers of the SC ultimately reflect a contribution from evaporated sweat, through the contribution of free AA derived from the keratinised lining of the acrosyringium. The significance of this contribution to the total NMF remains to be defined. Other sources of NMF i.e. lactate and urea were not determined in the present study and therefore any correlation between their occurrence/concentration and that of AA's in sweat cannot be established from the current data.

#### **NMF's as markers of skin condition**

Perturbations in the levels of filaggrin-derived NMF are associated with decreased SC integrity in both dry skin and more severe skin conditions such as AD. Depleted NMF are associated with abnormal barrier function, with levels significantly reduced in AD patients [10]. The clinical relevance of this observation is of even greater significance when considering the fact that disease severity has also been directly correlated with NMF levels, even in the non-lesional skin of AD patients [10]. Extensive studies including using RAMAN spectroscopy [30,31], in which the AA parameters are specifically studied and quantified, have indicated that measurement of these components of the NMF predict filaggrin genotype and can infer information on

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intragenic copy number [32]. Additionally, loss-of function mutations in the filaggrin gene are correlated with reduced NMF levels in the SC [33]. Previous results also demonstrated that the total free AA content of the SC is accurately and rapidly measured by a modified colorimetric method and correlates directly with the concentration of available PCA [34]. Given these facts and observations, we propose that a simple test measuring only the free total AA content of sweat (collected as described in this report), rather than a composite NMF picture including urea, lactate and PCA measurement, may provide a non-invasive diagnostic indicator of underlying loss of function mutations in the filaggrin gene associated with certain forms of AD.

## Conclusions

We describe for the first time the detailed free AA composition of freshly isolated ‘pure’ eccrine sweat, including some of the key derivatives of AA metabolism, from the same subject population. Our data strongly suggests that the free AA content of the ductal SC directly influences the composition of the AA’s encountered in eccrine sweat, and that the level and hydrolysis of the epidermal protein filaggrin, present within the acrosyringium is likely to be a major determining factor. AA’s play a key role in maintaining barrier integrity as they are the major source of skin derived NMF in the SC. Since NMF status is linked to filaggrin genotype, we propose the quantification of AA’s in sweat may serve as a rapid diagnostic biomarker of certain types of AD.

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## Figure Legends

### Figure 1.

Mean levels of pyrrolidine-5-carboxylic acid (PCA) and urocanic acid (UCA) ( $\mu\text{mol/l}$ ) in eccrine sweat as determined by GC/MS.

### Figure 2.

Comparison of the relative abundance of the top ten amino acids (and their derivatives) present in pure eccrine sweat compared to the profilaggrin protein (SwissProt accession number P20930), keratin I (SwissProt accession number P04264.6), loricrin (SwissProt accession number P23490), involucrin (SwissProt accession number P07476) and plasma [25,26]. Key to amino acids; ■ nonpolar and hydrophobic, ■ polar and hydrophilic, ■ acidic and ■ basic. Arg/Cit/Orn, sum of arginine, citrulline and ornithine, sum of all derivatives of arginine metabolism. Histidine/UCA, sum of all derivatives of histidine metabolism. Glutamine/PCA, sum of all derivatives of glutamine metabolism.

**Table I.** Free amino acid concentration ( $\mu\text{mol/l}$ ) in eccrine sweat from 12 healthy volunteers as determined by ninhydrin derivatisation followed by HPLC.

| Amino Acid             | Males ( $n=6$ ) |        |                | Females ( $n=6$ ) |        |                 | Combined ( $n=12$ ) |        | <i>P</i> males versus females |
|------------------------|-----------------|--------|----------------|-------------------|--------|-----------------|---------------------|--------|-------------------------------|
|                        | Mean            | SD     | Range          | Mean              | SD     | Range           | Mean                | SD     |                               |
| Taurine                | nd              |        |                | nd                |        |                 | nd                  |        |                               |
| Aspartic acid          | 63.8            | 35.4   | 21.4 - 107.0   | 119.6             | 79.0   | 27.3 - 213.0    | 91.7                | 65.3   | 0.181                         |
| Hydroxyproline         | nd              |        |                | nd                |        |                 | nd                  |        |                               |
| Threonine              | 112.1           | 75.2   | 41.6 - 241.0   | 209.0             | 61.6   | 145.0 - 295.0   | 160.5               | 82.8   | 0.083                         |
| Serine                 | 520.8           | 337.2  | 190.0 - 1110.0 | 984.0             | 301.1  | 794.0 - 1400.0  | 752.4               | 389.1  | 0.082                         |
| Glutamic acid          | 110.9           | 92.8   | 60.7 - 293.0   | 178.0             | 89.9   | 77.8 - 338.0    | 144.5               | 93.9   | 0.037*                        |
| Asparagine             | 16.1            | 6.4    | 8.6 - 25.2     | 38.0              | 22.5   | 15.3 - 74.4     | 27.1                | 19.5   | 0.073                         |
| Glutamine              | 12.0            | 7.9    | 4.1 - 19.6     | 17.8              | 13.0   | 5.8 - 35.3      | 14.9                | 10.7   | 0.360                         |
| Cysteine               | nd              |        |                | nd                |        |                 | nd                  |        |                               |
| Proline                | 34.9            | 22.2   | 14.1 - 67.6    | 72.8              | 26.4   | 41.9 - 112.0    | 53.9                | 30.6   | 0.057                         |
| Glycine                | 341.8           | 221.4  | 136.0 - 724.0  | 646.3             | 177.9  | 426.0 - 914.0   | 494.1               | 248.9  | 0.067                         |
| Alanine                | 196.8           | 132.3  | 74.9 - 437.0   | 387.0             | 88.4   | 285.0 - 510.0   | 291.9               | 146.2  | 0.065                         |
| Valine                 | 45.9            | 29.6   | 21.0 - 96.9    | 91.9              | 30.4   | 53.8 - 134.0    | 68.9                | 37.4   | 0.060                         |
| Cystine                | 0.9             | 0.7    | 0.2 - 2.1      | 1.2               | 0.6    | 0.4 - 1.9       | 1.1                 | 0.6    | 0.338                         |
| Methionine             | 3.4             | 2.1    | 1.1 - 6.5      | 8.5               | 3.9    | 2.9 - 13.2      | 6.0                 | 4.0    | 0.047*                        |
| Isoleucine             | 22.9            | 14.4   | 8.8 - 41.6     | 49.4              | 17.8   | 26.5 - 74.8     | 36.2                | 20.7   | 0.057                         |
| Leucine                | 32.4            | 16.7   | 12.8 - 57.8    | 62.5              | 26.4   | 25.6 - 92.9     | 47.5                | 26.3   | 0.101                         |
| Tyrosine               | 30.3            | 19.1   | 11.0 - 56.4    | 55.9              | 20.2   | 30.6 - 82.5     | 43.1                | 23.0   | 0.106                         |
| Phenylalanine          | 17.2            | 11.1   | 3.2 - 31.7     | 37.8              | 14.5   | 17.2 - 56.1     | 27.5                | 16.3   | 0.064                         |
| B-alanine              | 3.4             | 4.3    | 0 - 10.8       | 12.8              | 5.2    | 10.0 - 19.6     | 8.1                 | 6.7    | 0.043*                        |
| Ornithine              | 68.4            | 40.6   | 22.1 - 124.0   | 136.1             | 99.5   | 18.4 - 280.0    | 102.3               | 80.6   | 0.249                         |
| Lysine                 | 28.5            | 17.8   | 10.0 - 54.4    | 52.8              | 20.3   | 24.8 - 77.1     | 40.7                | 22.2   | 0.108                         |
| Tryptophan             | 7.1             | 4.8    | 2.4 - 14.5     | 15.1              | 5.6    | 8.4 - 22.9      | 11.1                | 6.5    | 0.083                         |
| Histidine              | 80.4            | 38.1   | 40.2 - 148.0   | 179.0             | 60.8   | 105.0 - 268.0   | 129.7               | 70.7   | 0.025*                        |
| Arginine               | 54.0            | 30.9   | 28.3 - 97.5    | 94.8              | 31.5   | 56.2 - 136.0    | 74.4                | 36.6   | 0.156                         |
| Citrulline             | 126.7           | 70.1   | 36.0 - 230.0   | 196.7             | 53.1   | 150.0 - 300.0   | 161.7               | 69.6   | 0.121                         |
| Total                  | 1930.6          | 1114.5 | 928.8 - 3754.5 | 3647.0            | 1151.7 | 2328.7 - 5310.5 | 3161.1              | 1403.9 | 0.063                         |
| Sweat rate (ml/20 min) | 1.28            | 1.24   | 0.41 - 2.94    | 0.77              | 0.33   | 0.32 - 1.25     | 1.03                | 0.9    | 0.367                         |

\*Males versus females  $P < 0.05$ .

