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High performance liquid chromatography tandem mass spectrometry dual extraction method for identification of green tea catechin metabolites excreted in human urine

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ABSTRACT

The simultaneous analysis of free-form and conjugated flavonoids in the same sample is difficult but necessary to properly estimate their bioavailability. A method was developed to optimise the extraction of both free and conjugated forms of catechins and metabolites in a biological sample following the consumption of green tea. A double-blind randomised controlled trial was performed in which 26 volunteers consumed daily green tea and vitamin C supplements and 24 consumed a placebo for 3 months. Urine was collected for 24 h at 4 separate time points (pre- and post-consumption) to confirm compliance to the supplementation and to distinguish between placebo and supplementation consumption. The urine was assessed for both free and conjugated metabolites of green tea using LC–MS^2 analysis, after a combination extraction method, which involved an ethyl acetate extraction followed by an acetonitrile protein precipitation. The combination method resulted in a good recovery of EC-*O*-sulphate (91 ± 7%), EGC-*O*-glucuronide (94 ± 6%), EC (95 ± 6%), EGC (111 ± 5%) and ethyl gallate (74 ± 3%). A potential total of 55 catechin metabolites were investigated, and of these, 26 conjugated (with methyl, glucuronide or sulphate groups) and 3 free-form (unconjugated) compounds were identified in urine following green tea consumption. The majority of EC and EGC conjugates significantly increased post-consumption of green tea in comparison to baseline (pre-supplementation) samples. The conjugated metabolites associated with the highest peak areas were *O*-methyl-EC-*O*-sulphate and the valerolactones M6/M6′-*O*-sulphate. In line with previous studies, EC and EGC were only identified as conjugated derivatives, and EGCG and ECG were not found as mono-conjugated or free-forms. In summary, the method reported here provides a good recovery of catechin compounds and is appropriate for use in the assessment of flavonoid bioavailability, particularly for biological tissues that may contain endogenous deconjugating enzymes.

1. Introduction

Green tea is the second most popular tea beverage, with the majority of consumption in Asian countries and North Africa, and with a recent increase in popularity in Western countries [1]. Green tea consists of catechins (or flavan-3-ols), which are a sub-group of the flavonoids. In comparison to other teas, green tea has the highest flavanol content (35–50%) [2], including catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (Fig. 1).

Daily consumption of green tea has been strongly associated with beneficial health effects, studied both in vivo and in vitro [3–7]. Epidemiological studies have linked green tea consumption with reducing the incidence of cancer onset [8], factors associated with cardiovascular disease [9–11] and factors related to the onset of diabetes [1,12]. Research has become more focused on the presence of conjugated metabolites in biological fluids following green tea interventions [13–15]. However, analysis of flavonoids in biological

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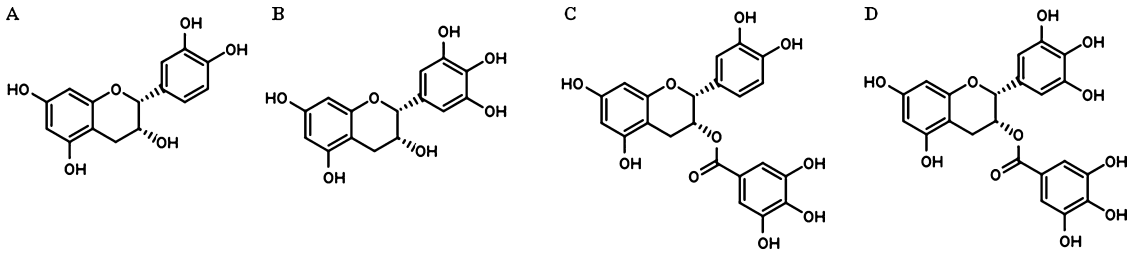

Fig. 1. The structures of the green tea catechins. (A) Epicatechin, (B) epigallocatechin, (C) epicatechin gallate and (D) epigallocatechin gallate.

samples presents several challenges. The free and aglycone forms of flavonoids are most soluble in lipophilic solutions, but are unstable during analysis [16–18]. Above pH 7 and 37 °C, the stability of free-form catechins is reduced due to degradation and epimerisation, and therefore, most free-form analysis utilises a low pH and low temperature environment with the addition of an antioxidant, such as ascorbic acid, to improve the stability [19–21]. As most of the conjugated catechins are commercially unavailable, enzyme deconjugation of green tea catechin metabolites present within biological samples [22–25] allows for the assessment of the metabolites relative to the free-form standards. However, the employment of this method has been questioned due to inefficient and incomplete hydrolysis [26], and as a result the more stable conjugated catechin forms have been assessed by monitoring the fragmentation patterns of the compounds using liquid chromatography mass spectrometry (LC–MS^2) analysis [27–31]. Due to the hydrophilic nature of the conjugated catechins, the majority of the methods that omit deconjugation utilise a protein precipitation of urine with acetonitrile prior to LC–MS^2 analysis [28,30]. A direct injection of the filtered urine sample following the consumption of green tea can also be employed, however, this can lead to ion suppression [32,33].

The extraction of free-form catechin metabolites in acetonitrile is associated with a poor recovery [28], and therefore, these molecules cannot be efficiently monitored in biological samples using extraction techniques that are suitable for the hydrophilic conjugated forms. In the present study, a combination of using an initial ethyl acetate extraction to isolate free-form metabolites followed by protein precipitation of the remaining urine sample with acetonitrile, which allows the identification of green tea conjugated metabolites, was utilised for the first time. This method is applicable for future human intervention studies in which both the free and conjugated forms can be monitored in biological samples following consumption of green tea, particularly for samples where β-glucuronidase and sulphatase are already present such as tissue extracts.

2. Experimental

2.1. Materials

Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), catechin (C) and taxifolin were purchased from Extrasynthése (Genay, France). Ethyl gallate and 3-methyl gallic acid were obtained from Apin Chemicals Ltd. (Oxfordshire, UK), hippuric acid, benzoic acid and 3-hydroxybenzoic acid were purchased from Fluka (Dorset, UK), ascorbic acid (AA) was from Sigma Aldrich (Dorset, UK) and syringic acid and gallic acid were purchased from Alfa Aesar (Lancashire, UK). All standards were of a HPLC purity (>90%). A Millipore Q water purifying system (Millipore, Hertfordshire, UK) was used to provide ultrapure water (≥18.2 MΩ cm at 25 °C) for LC–MS^2 analysis. EC-*O*-sulphate and EGC-*O*-glucuronide were synthesised using the method by Wong et al. [34].

2.2. Human intervention

In total, 50 volunteers enrolled on the 3-month double-blind randomised controlled trial conducted in the Photobiology Unit, Dermatology Centre, Salford Royal NHS Foundation Hospital (Manchester, UK). Ethical approval was received from the North Manchester Research Ethics Committee (reference 08/H1006/79) and the study conformed to the Declaration of Helsinki principles. Inclusion criteria for the study included white Caucasian adults (aged 18–65 years) with sun-reactive skin type I–II. Volunteers were excluded from the study if they had a history of skin cancer or photosensitivity, underwent sunbed use or sunbathing in the 3 months prior to the study, consumed photoactive medication or nutritional supplements, consumed >2 cups of tea per day or were pregnant or breastfeeding. The volunteers were allocated either placebo or green tea supplements that were the equivalent of 5 cups of green tea per day (5.25 mg C, 31.25 mg EC, 123.25 mg EGC, 65 mg ECG and 181.5 mg EGCG), along with 50 mg vitamin C to improve the gastrointestinal stability of the green tea supplements. The placebo and supplement were encapsulated and coded to maintain the blind aspect of the study. Urine was collected for 24 h at four time points during the intervention: baseline (pre-supplementation), day one (post-supplementation), week 6 and week 12. Urine was excreted into HCl-washed flasks containing AA (approximately 1 g/L) and was stored at −20 °C prior to analysis. Compliance to the study was tested through monitoring the presence of EC-*O*-sulphate and EGC-*O*-glucuronide excreted into urine during the trial at four time points, and by counting residual supplements.

2.3. Urine analysis

Urine was defrosted and 50 μL AA (6 mM in water, 1 mM final concentration in urine) and 50 μL ethyl gallate (internal standard; 4 μg/mL in water) were added to 200 μL urine (technical duplicates from one biological sample) in a 1.5 mL microcentrifuge tube (Eppendorf UK Ltd., Stevenage, UK) on ice. Samples were vortexed for 10 s before the addition of 500 μL ice cold ethyl acetate. Samples were then vortexed for 30 s and placed on ice for 2 min before centrifugation at 17,000 × *g* (4 °C) for 2 min. The extract (supernatant) was placed in a new microcentrifuge tube on ice. The process was repeated again and the extracts were pooled and dried down under nitrogen flow on ice. The dried down sample was then placed at −80 °C.

The urine sample (still on ice) remaining after the ethyl acetate extraction was then mixed with 800 μL ice cold acetonitrile and vortexed for 2 min before centrifuging at 17,000 × *g* (4 °C) for 10 min. The supernatant was placed into a pre-weighed microcentrifuge tube and the organic solvent was removed by centrifugal evaporation (settings: HPLC fraction, lamp off; Genevac, EZ-2 plus model; Genevac, Suffolk, UK). The microcentrifuge tube was