Antioxidant activity in human faeces

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(Received 10 May 1999 – Revised 24 February 2000 – Accepted 13 March 2000)

Scarcely-absorbed antioxidants might reach the large bowel and exhibit antioxidant activity, opposing the action of reactive O species by bacterial and cellular metabolism and thus contributing to protection from oxidative damage-induced gastrointestinal diseases. This study was carried out to evaluate the antioxidant activity in the faeces of a group of healthy subjects on a freely-selected diet, and to look for possible associations with the intake of some macro- and micronutrients and food groups. Fourteen subjects recorded their food intake three times for a period of 2 d, each time collecting all the faeces passed during the next 24 h. Total antioxidant activity (TAA; mmol 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox)/kg) of faecal suspensions was measured using the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$) decolorisation assay. The average TAA value of faeces was 26·6 (SD 10·2) mmol Trolox/kg wet faeces (range 7·5–50·5). The total amount of antioxidant equivalents excreted over 24 h, derived by multiplying the TAA by the amount of faeces passed over 24 h, was 3·24 (SD 1·51) mmol Trolox (range 0·92–5·82) and this was significantly correlated with the average 24 h intake of coffee ($P = 0{\cdot}002$), red wine ($P = 0{\cdot}034$), and particularly to the sum of coffee and red wine ($P < 0{\cdot}0001$). In conclusion, the faeces of healthy subjects show detectable capacity to scavenge radical cations, suggesting that antioxidant activity occurs in the colonic lumen. Moreover, such activity seems at least in part to be related to dietary habits.

Antioxidant activity: Faeces

There is currently great interest in the role of free radicals in the aetiology and pathogenesis of many degenerative diseases (Halliwell & Gutteridge, 1990), including gastrointestinal diseases (Thomson *et al.* 1998). The putative beneficial effects of antioxidant components of the diet, believed to oppose the deleterious action of free radicals, have been highlighted in a number of papers reporting data from epidemiological (Doll, 1990; Ames *et al.* 1993; Dragsted *et al.* 1993; Willett, 1994) and experimental (Fuhrman *et al.* 1995; Visioli *et al.* 1995; Serafini *et al.* 1996) studies. Recently, total antioxidant intake has been estimated by determining the oxygen radical absorbance capacity of vegetable foods consumed in mixed diets, and was found to be related to an increase of antioxidant activity in blood compartments (Cao *et al.* 1998). However, many food components which manifest antioxidant capacity *in vitro*, such as polyphenols present in fruits and beverages, are likely to be scarcely absorbed, as suggested by ileostomy balance studies (Hollman *et al.* 1995) and the recovery in rat faeces (Bravo *et al.* 1993). Therefore, we hypothesise that faeces, taken as a marker of intestinal contents, have a detectable antioxidant activity, and that foods containing scarcely-absorbed dietary antioxidants may substantially modify the activity. This could have potential implications in terms of colonic health and contribute to clarification of the effect of diets high in antioxidants. Over the past few years, several methods have been developed for measuring the total antioxidant capacity of biological fluids, blood components, and food and beverages (Cao *et al.* 1993, 1996; Miller *et al.* 1993; Ghiselli *et al.* 1995; Wang *et al.* 1996), but to our knowledge, none have been applied to faeces. The objective of this study was to measure the total antioxidant activity (TAA) of human faeces from healthy subjects on a freely-selected diet and to relate it to food intake.

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; TAA, total antioxidant activity; TA-24 h, total antioxidants excreted over 24 h; Trolox, 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid.

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Materials and methods

Chemicals

6-Hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 10 mM-PBS pH 7·4, and manganese dioxide were purchased from Sigma-Aldrich (Sigma-Aldrich srl, Milan, Italy).

Subjects

Fourteen healthy subjects (six males, eight females), aged 36 (SD 11) years (range 23–54) with BMI 21·8 (SD 2·4) kg/m^2 (range 18·7–26·9) took part in the study. None of them had been on antibiotic treatment or other therapy for at least 4 weeks prior to the study. No dietary supplements, laxatives or other cathartics were used during the test days. The participants were omnivorous and were encouraged to keep their diets, alcohol intakes, smoking and exercise patterns constant over the period of the study. No subject had a history of gastrointestinal disease.

Protocol

During the period April–June 1997, on three different occasions, the subjects recorded the weight of all the food eaten in a 48 h dietary diary. They then collected all the faeces passed during the next 24 h into sterile plastic bags, which were kept refrigerated and carried to the laboratory within the following day. Specimens collected by the same subject over the 24 h were mixed and the resulting pooled sample was weighed. An aliquot was taken to determine DM and a second aliquot was used to prepare a faecal suspension (50 g/kg PBS). Centrifugation at 8000 ***g*** for 15 min was performed to obtain a supernatant free of solid cellular debris. This supernatant was divided into portions and frozen at −80°C.

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation decolorisation assay

After thawing, supernatants were assayed for their TAA using the ABTS$^{\bullet+}$ decolorisation assay described by Miller & Rice-Evans (1997) using a Cary-5 Spectrophotometer (Varian, Cary, Australia). The final absorbance reading was taken 1 min from the start of the reaction. Faecal values of TAA (mmol Trolox/kg wet faeces) were obtained by multiplying the TAA of the faecal supernatant by the dilution. The amount of total antioxidants excreted over the 24 h (TA-24 h; mmol) was derived by multiplying the TAA by the amount of faeces passed over the 24 h. For each subject, results of the three experiments were averaged.

Food records

The calculation of the composition of the diets in a 24 h period was performed dividing by two the results of the analysis of each 48 h dietary diary. Results of the three experiments were then averaged in order to obtain a mean daily value of nutrients and food intake for each individual subject. Nutrient composition was derived using Italian standard food tables (Carnovale & Marletta, 1998). Individual foods were also considered and divided into three main classes: total vegetables, total fruits, and total beverages. Each class was further divided into subclasses, taking into account their putative content of dietary antioxidants: fruits or vegetables rich in phenols or in carotenoids, beer, red wine, coffee, tea and fruit juices.

Statistical analysis

Data are reported as means and standard deviations. The relationships between faecal weight, TAA, TA-24 h and intake of micro- and macronutrients and groups of foods were investigated by Pearson's univariate correlation analysis. Correlations were then recalculated by controlling for the influence of the specific variables that presented the highest correlation coefficient, using the partial correlation facility of the statistical package SPSS 9·0 (SPSS Inc., Chicago, IL, USA); P values $<0·05$ were considered significant.

Results

All the faecal supernatants had detectable antioxidant activity; seven samples needed a further dilution, as their concentration was too high for the conditions of the test. The faecal wet weight and the percentage DM, TAA of faeces and the amount of antioxidants excreted over 24 h are reported in Table 1. The composition of the diets consumed by the subjects and the intake of antioxidant-rich foods are reported in Tables 2 and 3 respectively. In Table 4, the coefficients of correlation and the levels of significance of the univariate relationships between food intake and the faecal variables are reported. Total fruits, fruits and vegetables rich in phenols, and coffee were positively and significantly correlated with faecal weight. However, for the vegetables rich in phenols, the significance disappeared after controlling for the intake of coffee and red wine. TAA was not correlated with any food group, whereas a direct and significant correlation was found between TA-24 h and the mean daily intake of coffee ($P = 0·002$), red wine ($P = 0·034$), and particularly the sum of the two beverages ($P < 0·0001$). Controlling for the intake of coffee and red wine confirmed these relationships as the only ones which were significant. Finally, none of the faecal variables were significantly correlated to micro- and macronutrients (data not shown).

Table 1. Faecal weight, DM, faecal total antioxidant activity (TAA) and total antioxidants excreted over 24 h (TA-24 h) in subjects on a freely-selected diet*

(Mean values, standard deviations and ranges for fourteen subjects)

	Mean	SD	Range
Faecal wet weight (g)	129·0	57·2	61·2–264·2
Faecal DM (%)	23·3	5·2	15·8–31·2
TAA (mmol Trolox /kg wet faeces)	26·6	10·2	7·5–50·5
TA-24 h (mmol Trolox)	3·24	1·51	0·92–5·82

Trolox, 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid.
* Subjects repeated a 24 h faecal collection three times. For details of analytical procedures see p. 706.

Table 2. Average daily intake of energy and nutrients from 48 h food diaries repeated three times*

(Mean values, standard deviations and ranges for fourteen subjects)

	Mean	SD	Range
Energy			
kcal	2003	437	1092–2923
MJ	8·380	1·828	4·569–12·230
Protein (g)	73·9	15·9	47·5–96·3
Total fat (g)	75·6	18·4	39·4–104·5
Saturated fat (g)	27·8	8·1	12·7–41·9
Polyunsaturated fat (g)	9·0	3·3	4·6–13·8
Total carbohydrate (g)	260·0	74·1	145·0–411·5
Sugars (g)	92·6	44·6	32·8–178·4
Total starch (g)	155·2	40·3	90·4–217·7
Resistant starch (g)	6·0	1·8	3·8–10·2
Total dietary fibre (g)	17·5	5·6	11·3–32·5
Soluble fibre (g)	6·7	2·1	4·0–11·1
Insoluble fibre (g)	10·4	3·7	6·7–21·0
Alcohol (g)	7·5	7·3	0·0–22·8
Calcium (mg)	885	296	379–1240
Phosphorus (mg)	1278	286	768–1732
Sodium (mg)	2651	1054	692–4278
Potassium (mg)	2730	839	1913–4597
Iron (mg)	11·1	2·2	7·7–14·6
Thiamin (mg)	0·77	0·21	0·51–1·17
Riboflavin (mg)	1·37	0·33	0·76–1·85
Niacin (mg)	23·9	6·6	9·7–35·7
Vitamin A (μg retinol equivalents)	1594	2913	451–11688
Ascorbic acid (mg)	106	76	41–315

* Nutrient composition was calculated from the Italian standard food tables (Carnovale & Marletta, 1998).

Discussion

A vast literature concerning the possible role of oxidative stress in the pathogenesis of gastrointestinal diseases has accumulated over recent years. Dietary factors, the intestinal microflora and endogenously produced metabolites contribute to the generation of reactive species of O and N in the colon. Babbs (1990) has demonstrated that dilution of faeces can produce hydroxyl radicals, as a result of the bacteria oxidative metabolism and the catalytic action of available Fe. Minor inflammatory events can be another source of free radicals generation in the large bowel. Indeed, activated neutrophils and macrophages elaborate reactive O species when migrating in injured tissues (Babior, 1978) and this may be responsible for cellular damage in inflammatory processes. Moreover, bile acids can affect the gut oxidative status inducing phospholipid breakdown in the membrane of colonic cells (Craven *et al.* 1986). The release of arachidonate results in the activation of the enzymes lipoxygenase and cyclooxygenase with the production of free radicals. Free radicals can damage several crucial biological components, including proteins, DNA and membrane lipids. In addition, the hydroxyl radical participates in aromatic hydroxylations (Grootveld & Halliwell, 1986); this can be particularly dangerous in the colon where a variety of hazardous chemicals, either derived from drugs, or contained in food as additives and constituents, are susceptible to be converted through this mechanism into carcinogens. Different diets might affect the production of free radicals in the colon. A recent study has demonstrated that the potential for hydroxyl radical formation in the faeces is markedly enhanced when consuming diets considered a risk factor for colon cancer (i.e. rich in fat and meat and low in fibre) (Erhardt *et al.* 1997). However, oxidative stress occurs only if pro-oxidants are in excess of antioxidants.

Table 3. Average daily intake of antioxidant-rich foods for 48 h food diaries repeated three times

(Mean values, standard deviations and ranges for fourteen subjects)

	Mean	SD	Range
Total vegetables (g)	233	130	49–523
Total fruits (g)	161	160	45–667
Total beverages (g)	318	208	30–703
Vegetables rich in phenols (g)	136	99	32–450
Vegetables rich in carotenoids (g)	176	119	20–493
Fruit rich in phenols (g)	123	155	0–633
Fruit rich in carotenoids (g)	66	53	15–192
Tea (g)	113	158	0–450
Fruit juices (g)	19	32	0–102
Beer (g)	53	66	0–208
White wine (g)	9	20	0–58
Red wine (g)	45	59	0–170
Coffee (g)	78	90	0–338
Coffee+red wine (g)	124	112	0–338

Antioxidants have been shown to be helpful in reducing responses in inflammatory bowel diseases (Reimund *et al.* 1998) and the beneficial effect of salicylate, one of the more effective drugs for the treatment of ulcerative colitis, has been also attributed to its capacity to counteract the oxidation products (Pearson *et al.* 1996). In rats, oral administration of rutoside ameliorated inflammatory diseases, possibly through the prevention of glutathione depletion (Cruz *et al.* 1998) and in man, phytate and vitamin E has been suggested as preventing colon cancer thanks to their antioxidant properties (Graf & Eaton, 1993; Stone & Papas, 1997). A variety of endogenous substances, such as sulfated glycoproteins, uric acid, coproporphyrins and other bile pigments, which in certain conditions exhibit antioxidant activity (Stocker *et al.* 1990; Williams *et al.* 1994), are already present in the intestinal lumen and their concentration may indeed be modulated by several components of the diet. Nevertheless, poorly absorbed dietary antioxidants, such as insoluble polyphenols (highly polymerised or bound tannins common in foods of plant origin (legumes, cereals, fruits) and beverages (tea, cider, wine)) are very likely to reach the colon possibly hampering oxidative reactions. Recently, Hagerman *et al.* (1998) speculated that tannins, which are resistant to degradation by intestinal enzymes, might remain in the digestive tract, protecting biomolecules from possible oxidative damage occurring during digestion, therefore sparing other antioxidants and contributing to the enhancement of the whole antioxidant status of tissues. Our results indicate that faeces do have a remarkable antioxidant activity (26·6 (SD 10·5) mmol Trolox/kg), much greater than plasma (1·46 (SD 0·14) mmol Trolox/l) (Rice-Evans & Miller, 1994) and that the total amount of antioxidants excreted over 24 h is significantly and positively related to the consumption of beverages rich in phenols. In order to study the correlation between excretion of antioxidants and food intake, we repeated 2 d weighed records of all food consumed three times. The weighed dietary record is considered the most accurate method of dietary assessment

Table 4. Summary of the coefficients of correlation (*r*) and levels of significance (*P*) of the univariate relationships between food intake and the faecal variables

Food classes	Faecal weight (g)		TAA (mmol Trolox/kg wet faeces)*		TA-24 h (mmol Trolox)†	
	r	*P*	*r*	*P*	*r*	*P*
Total beverages	0·332	0·246	−0·047	0·874	0·387	0·172
Red wine	0·299	0·299	0·211	0·468	0·569‡	0·034
Beer	0·324	0·258	−0·307	0·285	0·099	0·736
Tea	−0·192	0·512	−0·089	0·762	−0·152	0·603
Coffee	0·668	0·009	0·213	0·465	0·758§	0·002
Fruit juices	−0·105	0·721	−0·094	0·750	−0·101	0·732
Coffee + red wine	0·696	0·006	0·283	0·327	0·911	<0·0001
Total vegetables	0·342	0·232	−0·048	0·870	0·306	0·287
Vegetables rich in carotenoids	0·467	0·093	−0·020	0·945	0·414	0·141
Vegetables rich in phenols	0·659‖	0·010	−0·130	0·657	0·471	0·090
Total fruits	0·698¶	0·006	−0·212	0·467	0·445	0·111
Fruits rich in carotenoids	0·039	0·895	−0·106	0·719	−0·090	0·760
Fruits rich in phenols	0·720**	0·004	−0·168	0·565	0·506	0·065

TAA, total antioxidant activity; Trolox, 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid; TA-24 h, total antioxidant excreted over 24 h.
* TAA of faecal suspension measured by 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorisation assay (see p. 706).
† TA-24 h was derived by multiplying the TAA by the amount of faeces passed over the 24 h.
When the correlations were recalculated by controlling for the influence of ‡coffee, §red wine, ‖,¶,**red wine+coffee, then the coefficients of correlation (*r*) and levels of significance (*P*) were: ‡*r* 0·8656, $P < 0·001$; §*r* 0·7760, *P*=0·002; ‖*r* 0·4247, $P = 0·169$; ¶*r* 0·6034, *P*=0·038; **r* 0·5980, *P*=0·040. For details of statistical procedures see p. 706.

(Bingham *et al.* 1995). Nevertheless, partly due to the very low intake reported by one female subject (4·57 MJ 1092 kcal), the resultant average energy intake was rather low for a mixed group of free-living subjects (8·38 MJ, 2003 kcal), although not very different from that reported in Italy from 1994 to 1996 by the National Institute of Nutrition of 8·7 MJ (2078 kcal) (Turrini *et al.* 1999). Moreover, based on the age and the anthropomorphic characteristics of the subjects, the mean value of energy intake:BMR was 1·3. This value is greater than the cut-off (1·27) corresponding to the lowest plausible energy intake (Hirvonen *et al.* 1997). However, it cannot be excluded that some under-reporting might have occurred, being a common problem in dietary analysis studies (Briefel *et al.* 1997; Pryer *et al.* 1997). In principle, under-reporting, when due to under-recording of food intake, might affect the interpretation of a causal relationship between variables by weakening correlations. However, in our case it is possible that under-reporting of food intake might have been associated with true undereating, as already shown in a group of highly-motivated lean women during a weighed dietary record (Goris & Westerterp, 1999). Since undereating should not affect correlations, it is likely that in our group of subjects, the effect of dietary components on the total amount of antioxidants excreted over 24 h was truly limited to beverages rich in phenols.

Bioavailability of phenols present in foods and beverages is still controversial, being a function of their basic structure, the degree of glycosylation–acylation, conjugation with other phenolics, molecular size, degree of polymerization, and solubility (Bravo, 1998). Our data bring indirect evidence of incomplete absorption of phenolic antioxidants present in coffee and red wine. Moreover, the remaining antioxidant activity of polyphenols in the faeces seems to confirm their resistance to bacterial degradation observed *in vitro* (Bravo *et al.* 1993). The independence of TAA (i.e. the concentration of antioxidants in faeces) from the intake of coffee and red wine, and the fact that coffee intake was related to stool weight (r 0·668, $P = 0·009$) could support the hypothesis of an homeostatic mechanism controlling faecal mass. Similarly, it has been shown that tea consumption affects the faecal mass both in human subjects (Bingham *et al.* 1997) and in rats (Bravo *et al.* 1994). This has been attributed to the fact that insoluble polyphenols, which are not broken down in the upper part of the gut and reach the colon, significantly increase water, fat and protein excretion, causing an increase in total faecal weight (Bravo *et al.* 1993). The fact that coffee is related to faecal mass and supplies antioxidants to the colon is of particular importance, since high coffee consumption was repeatedly associated with a reduced risk of developing colon cancer in affluent countries (Potter, 1996).

The results of this observational study encourage further detailed studies on the presence and role of dietary antioxidants in the intestinal lumen. In particular: (1) balance experiments are needed to assess the quantity and activity of unabsorbed antioxidants; (2) the antioxidant activity of the intestinal contents should be considered when assessing the effects of dietary components on bowel diseases, including cancer.

Acknowledgement

This study was supported by CNR, National Research Council of Italy, Contract no. 95·00773·PF41.

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Appendix

Fruits and vegetables consumed by subjects divided by classes

The classification is based on direct analysis (DiSTAM internal food database; Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Milan, Italy) and on data by Macheix *et al.* (1990), Hertog *et al.* (1992, 1995), Mangels *et al.* (1993), Scott & Hart (1995), Granado *et al.* (1996), Peterson & Dwyer (1998), King & Young (1999), Pillow *et al.* (1999), Wakai *et al.* (1999).

Vegetables rich in carotenoids:
- Asparagus
- Carrot
- Legumes
- Lettuce
- Rocket salad
- Spinach
- Sweet Corn
- Sweet Pepper
- Tomato

Fruits rich in carotenoids:
- Apricot
- Cantaloupe
- Loquat
- Kiwi
- Pink Grapefruit
- Watermelon

Vegetables rich in phenols:
- Celery
- Legumes
- Lettuce
- Onion
- Tomato
- Soyabean sprouts
- Aubergine

Fruit rich in phenols:
- Apple
- Cherry
- Plum
- Raspberry
- Strawberry
- Banana
- Grapefruit
- Kiwi
- Orange
- Peach
- Olive

Other vegetables:
- Cucumber
- Fennel
- Radish, red
- Zucchini