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Development of a questionnaire and a database for assessing dietary D-limonene intake

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Abstract

Objective: Increasing recognition of the potential importance of phytochemicals in the aetiology of cancer and heart diseases has highlighted the need for methods to measure individual phytochemical consumption that are sufficiently simple to be used in large epidemiological studies and whose reproducibility and accuracy have been quantified. D-Limonene is a natural component of a variety of foods and beverages and is found mainly in citrus fruits. However, D-limonene is not assessed by any nationally available analysis database.
Design: We designed our study to assess the D-limonene content of different citrus juices and beverages and to develop a dietary assessment instrument to measure consumption of citrus foods (fruit, juice and peel) and D-limonene intake and test it for reliability.
Subjects and methods: A total of 120 citrus juice samples were analysed and used to develop the preliminary D-limonene database. A self-administered citrus food-frequency questionnaire was developed and administered twice to participants, separated by a 2-month interval. The questionnaire was tested for reproducibility of estimates of citrus food consumption and D-limonene intake among 120 participants.
Results: Correlation coefficients between the two administrations of the questionnaire ranged from 0.50 for citrus peel use to 0.82 for orange juice. Mean intakes (range) of D-limonene from citrus juices among consumers were 13.0 (0.24–141.9) mg day^{-1} and 13.2 (0.07–83.9) mg day^{-1} ($r = 0.60$, $P < 0.001$).
Conclusion: The citrus frequency questionnaire developed in this study provided highly reproducible estimates of citrus foods, citrus peel and D-limonene intakes. This instrument may be a useful tool in studies of the associations between citrus peel use, D-limonene intake and risk for chronic disease.

Keywords
Limonene
Citrus
Questionnaire
Reliability
Database

For the past two decades, epidemiologists have observed lower risks of cancer and cardiovascular diseases in populations that frequently consume fruits and vegetables. Efforts to identify the anticancer agents in fruits and vegetables have focused on the micronutrients common to these foods, such as vitamins A, C and E, and the carotenoids. More recently, interest has focused on the potential anticancer role of phytochemicals, non-nutrient components of fruits and vegetables. Non-nutrient compounds belonging to more than 12 separate categories of chemicals have been found to exert inhibitory effects in experimental carcinogenesis[1–3]; however, clearly defined data relating these experimental findings to epidemiological studies of the relationship of diet to cancer in man are not conclusive.

Numerous phytochemicals have been shown to be anticarcinogenic under experimental conditions and may account for at least part of the cancer-prevention effects of fruit and vegetable consumption. D-Limonene, a monocyclic monoterpene, is a natural component of a variety of foods and beverages and is found in many fruits (especially citrus fruits), vegetables, meats, spices and other food items[4]. The principal sources of D-limonene are the oils of orange, grapefruit and lemon[5]. D-Limonene has efficacy in pre-clinical models of breast[6–8], skin[9], liver[10], lung and forestomach[11,12], gastric[13] and colon[14] tumours. As a result, its cancer chemotherapeutic activities are under evaluation in Phase I therapeutic clinical trials[15,16].

A conceptual limitation that has plagued epidemiological studies has been the approach to analysis. In most studies, food intake data have been converted to summarised nutrients, which have been the focus of the risk analysis. Thus, the limitations of food composition databases have placed important limits on what could be analysed. In addition, in studies that have analysed food intake by food groups as well as by nutrients, food groups have generally been found to have greater relative risks (or relative protection) associated with them than specific nutrients. This may be due both to the presence of important compounds (phytochemicals) not included in standard food composition databases and to the importance of combinations of factors present in whole foods.

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Increasing recognition of the potential importance of phytochemicals in the aetiology of cancer and heart diseases has highlighted the need for methods to measure individual phytochemical consumption that are sufficiently simple to be used in large epidemiological studies and whose reproducibility and accuracy have been quantified. This study was designed to assess the D-limonene content of different citrus juices and beverages and to develop a specific citrus questionnaire to assess usual citrus peel, pulp and juice consumption. The consumption data could then be converted to mg of D-limonene per day, using these newly developed food composition data.

Methods

Identifying D-limonene levels in citrus juices and beverages

Beginning in April 1998, we conducted an extensive survey of the various citrus juice products available in Tucson, Arizona. D-Limonene was assessed in 120 samples of citrus juice products. Commercial juice samples were purchased from three different local stores. An attempt was made to collect different brands, different storage containers, same brand stored and sold in different containers, orange juice with and without pulp from the same brand and same type of storage container, and a variety of mixed juices containing citrus. All of the samples were purchased one to two days prior to preparation for analysis. All of the original labels were removed and the juices were numerically coded and sent to the laboratory for analysis. The juice samples from concentrate were prepared by completely mixing the content of one can of concentrate with three cans of water. All analyses were completed within three weeks. To date a total of 120 citrus juice samples have been analysed and the detailed methodology is described elsewhere[17]. Seventy-five samples were analysed at the Arizona Cancer Center[17] and 48 samples were analysed at the laboratory of Craft Technologies, Inc. (Wilson, NC). Quality control samples revealed a variation of 8–10% in D-limonene levels between the two laboratories. This variation might be attributed to the difference in methodology, gas chromatography–mass spectrometry vs. high-performance liquid chromatography, or the batch number of the juice analysed.

Development of the Arizona Citrus Questionnaire

We designed a detailed citrus questionnaire as a supplement to the Arizona Food Frequency Questionnaire (AFFQ) with the objective of categorising individuals by intake of D-limonene. The Arizona Citrus Questionnaire (ACQ) was developed after a series of focus groups identified usual patterns of citrus consumption. The questionnaire sought information on average citrus fruit and juice intakes over the past year as well as how the juice was stored, i.e. types of containers. Information was also sought for the addition of citrus juices and citrus peel during food preparation and/or food serving. Subjects were asked to indicate how often, on average, they consumed the specified amount of each citrus product during the past year. There were eight possible responses, ranging from never to 3 or more times per day. We then modified the ACQ and added a question about frequency of consumption in season. For each juice, we specified a medium serving size, and participants could mark their usual serving size as small, medium or large. For each fruit, participants could select from a portion size of a half fruit up to 2 or more fruits; while for citrus peel, they were asked to identify the number of tablespoons used and/or consumed or the number of fruits peeled. The questionnaire contained blank spaces for filling in different uses of citrus peel. The ACQ is now available in a format that is optically scanned to provide raw computer files for subsequent analysis. On average it took the subjects 7 minutes to fill out the questionnaire.

Study design and data collection

In 1998, the questionnaire was tested for short- (1-week) and long-term (6-month) reliability within a randomly selected sample of men and women between the ages of 40 and 80 years ($n = 40$) who had participated in a larger population-based epidemiological study on skin cancer[17]. They all completed interviews at baseline, 1 week and 6 months. The reproducibility of the frequency categories chosen by the subjects was analysed by examining the agreement among the three administrations of the ACQ. Reliability for each of the specific questions was very high even at 6 months. The reliability results are published elsewhere[17].

The citrus questionnaire has been also tested for reliability among 120 healthy subjects (aged 40–82 years) participating in a 6-month skin biomarker study. Data collection started in February 1999 and took 12 months. The ACQ was self-administered (during the participants'

Table 1 Timing of administration of the dietary assessment methods used to evaluate the reproducibility and comparability of citrus intake

	Baseline	1 week	2 months	4 months	6 months
Interviewer-administered	ACQ	ACQ			ACQ
Self-administered	AFFQ			ACQ	ACQ

ACQ – Arizona Citrus Questionnaire; AFFQ – Arizona Food Frequency Questionnaire.

scheduled visits) on two occasions, separated by a 2-month interval. All 120 subjects completed the AFFQ and the ACQ. The AFFQ was administered at baseline (month 1), while the ACQ was administered at month 4 and month 6 (Table 1). We also compared the data collected from the citrus questionnaire with that drawn from the baseline AFFQ.

Processing the data

Coding, entry and analysis of the questionnaires were conducted by the Nutrition Core at the Arizona Cancer Center. The quality control/quality assurance (QC/QA) procedures include 25% duplicate entry. All coders go through a formal training and probationary period until their work meets Core standards. The AFFQ is optically scanned to provide raw computer files for subsequent analysis. The QC/QA procedure is to double-scan each AFFQ. To avoid having to write new analysis programs for each modification or variant, generic food-frequency analysis software has been written. The program consists of three subprograms, all of which run on IBM-PC class machines. The first program reads a command file, and creates all files required for a specific analysis of a specific form of the AFFQ. The second program, using files created by the first, can be used to convert any number of scanned output forms into a file containing gram weight/day data for all items on the AFFQ. The third program converts the gram weight/day into nutrients, using an expansion and enhancement of the Block nutrient database. This software makes it possible to analyse any set of lines. We eventually shall use this flexibility to examine specific foods in relation to D-limonene. The totals of all nutrients are always reported, and subtotals within food groups can also be requested. The food groups are defined by flags in the nutrient database, and subtotals in any combination of groups can be requested.

Development of D-Limonene Database and the computer program

Nationally available nutrient analysis databases do not assess D-limonene; therefore we analysed 120 samples of citrus juices and beverages and developed a preliminary database for D-limonene at the Arizona Cancer Center. This preliminary D-limonene database is the first step in the development of the D-Limonene Database and is based on analysis of citrus juices and beverages. The 120 citrus juices analysed to date include orange juice, grapefruit juice, lemonade, limeade, lemon and lime juices, and mixed juices with citrus ingredients. The juices were selected after researching availability to consumers at grocery stores and fast food restaurants in Tucson, Arizona. After we had identified and analysed all of the citrus juice samples, D-limonene levels were averaged by type of container for each juice.

We were able to develop a computer analysis program to routinely connect dietary data from the ACQ and the upgraded nutrient database, to assess D-limonene intake. Currently, only citrus juices are processed. Forms are entered using a screen-input program written in SAS[18]. Subsequent quality control and nutrient breakdown (using a SAS program) occur in the following steps for each juice (orange, grapefruit, lemonade and limeade). First, frequency of use over the past year is converted to average times/day. The method used in determining portion size in grams is modelled after that used in the Block database[19]. This method uses sex, age and portion size (small, medium or large) to determine the final gram weight of a particular food. In cases where portion size was missing, medium was assumed. Second, a preparation/container code is determined using the following hierarchy (in this order): (1) fresh at home, (2) smoothie, soda, thirst quencher, Hi-C, (3) frozen concentrate, (4) ready-made from concentrate, glass container, (5) ready-made from concentrate, can container, (6) ready-made from concentrate, plastic container, (7) ready-made from concentrate, carton container, (8) if container is not indicated we use an average of all types. Third, average grams per day are calculated. Fourth, using the juice and preparation/container codes, the limonene database is accessed to determine the $\mu g\,ml^{-1}$ of limonene. Last, the $g\,day^{-1}$ consumed is then used to determine the $\mu g\,day^{-1}$ of limonene consumed.

Two datasets are then created: a detail and a summary file. The detail file of dietary limonene ($\mu g\,day^{-1}$) contains one record for each juice for each participant. The summary file for dietary limonene ($\mu g\,day^{-1}$) contains the sum of limonene per day from each juice. That is one record for each participant.

Statistical analyses

The reproducibility of the ACQ is expressed as Pearson (for frequency), Spearman (for portion size) and kappa (for container/preparation) correlation. For each administration of the questionnaire, the percentage of participants who consumed each type of citrus food as well as the median (25th and 75th percentiles) grams per day among consumers were calculated. Pearson and Spearman correlation coefficients between the intake estimates ($g\,day^{-1}$ for citrus foods and $mg\,day^{-1}$ for D-limonene) based on the first administration of the citrus questionnaire (ACQ1) and those based on the second administration of the citrus questionnaire (ACQ2) and the AFFQ were calculated. Precision was examined using intra-class correlation coefficients between estimates of D-limonene from the second administration of the questionnaire.

To compare absolute citrus food intakes, sample medians and 25th and 75th percentiles were computed. Reproducibility and relative validity (comparability) at the citrus food level were assessed by examining differences in distribution of intake between ACQ1, ACQ2 and AFFQ. The sign test and Wilcoxon signed-rank test were used to test whether differences in distributions were statistically

significant, defined as two-sided *P*-values of 0.05. Because both results were identical, we report the sign test results. All statistical analyses were carried out using STATA computer software[20].

Results

Characteristics of the study population are shown in Table 2. The mean age of study participants was 60 years and the average body mass index was 30 kg m^{-2}.

The reproducibility of the ACQ, expressed as Pearson (for frequency), Spearman (for portion size) and kappa (for container/preparation) correlations, is presented in Table 3. Overall, the 2-month and 6-month reproducibility of the ACQ was highly significant. The reproducibility of the interviewer-administered ACQ ranged from 0.59 for other citrus fruits to 0.96 for orange juice, while the reproducibility of the self-administered ACQ ranged from 0.36 for other citrus fruits to 0.82 for orange juice.

Descriptive information regarding citrus food consumption during the past year is presented in Table 4. On average, 70% of the participants reported consumption of orange and orange juice, 40 to 65% consumed grapefruit and grapefruit juice, and 15% consumed citrus peel. Correlation coefficients for the g day^{-1} of citrus juices and fruits between the two administrations of ACQ ranged from 0.45 for grapefruit to 0.60 for orange juice. Overall, Pearson and Spearman correlation coefficients comparing citrus food intake (g day^{-1}) based on the second administration of ACQ and AFFQ with that based on the first administration of ACQ were statistically significant ($P \leq 0.05$).

Daily median intakes for five common citrus foods assessed by ACQ1, ACQ2 and AFFQ are also given in Table 4. For these citrus foods the questionnaire estimates were based on reported frequencies and reported portion sizes. Portion sizes are based on the actual number of fruit(s) consumed (from 1/2 fruit to >2 fruits) for the ACQ, while they are based on the three categories – small, medium or large – for the AFFQ. The median estimate of grapefruit intake according to the AFFQ was comparable to that based on ACQ1 and ACQ2.

Estimates of D-limonene intake among citrus juice consumers were nearly identical for the two administrations of the questionnaire (Fig. 1). D-Limonene intake (mean ± standard deviation) was 13.0 ± 20.2 and 13.2 ± 16.5 mg day^{-1}, as estimated by the first and second administration of the ACQ. The Pearson and Spearman correlation coefficients for the comparison between the two administrations were 0.60 and 0.72, respectively, while the intra-class correlation was 0.84.

Daily median intakes for D-limonene (mg day^{-1}) from citrus juices assessed by ACQ1 and ACQ2 are shown in Table 5. For these citrus juices the D-limonene estimates were based on reported frequencies, portion sizes and type of juice container. Spearman correlation coefficients for mg day^{-1} of D-limonene between the two administrations of the ACQ ranged from 0.58 to 0.72.

Table 2 Demographic and behavioural characteristic of participants who completed the reliability and validity studies of the Arizona Citrus Questionnaire ($n = 120$)

Characteristic	Mean ± SD or %
Age (years)	60.1 ± 10.1
Male (%)	47.5
Married (%)	66.9
Education	
At least some college (%)	84.5
Smoking (%)	
Never	36.7
Former	53.7
Current	9.5
Drink alcohol (%)	64.6
Body mass index (kg m^{-2})	30 ± 5.65
Energy intake	
kcal day^{-1}	1833.61 ± 787.61
Percentage of kcal from fat	33.09 ± 10.89

SD – standard deviation.

Table 3 Correlation coefficients (r) between citrus intake estimates based on repeated administrations of the Arizona Citrus Questionnaire (reproducibility)†

	Reproducibility ($n = 120$)‡ (self-administered)	Reproducibility ($n = 40$)§ (interview)	
Citrus group	2-month¶	1-week¶	6-month¶
Orange juice			
Frequency	0.82*	0.99*	0.96*
Portion size	0.41*	0.92*	0.88*
Container/preparation	0.68*	1.00*	0.81*
Grapefruit juice			
Frequency	0.78*	1.00*	0.84*
Portion size	0.55*	0.99*	0.96*
Container/preparation	0.68*	0.90*	0.53**
Lemonade			
Frequency	0.73*	0.93*	0.84*
Portion size	0.55**	0.96*	0.86*
Container/preparation	0.72*	1.00*	0.69*
Orange			
Frequency	0.54*	0.96*	0.91*
Portion size	0.31**	0.94*	0.68*
Grapefruit			
Frequency	0.54*	0.94*	0.87*
Portion size	0.41**	1.00*	0.81*
Citrus peel			
Frequency	0.48**	0.87*	0.81*
Marmalade			
Frequency	0.62*	0.93*	0.80*

† Spearman rank correlation coefficients between citrus intake estimates based on repeated administration of the Arizona Citrus Questionnaire (ACQ) (reproducibility).
‡ Two administrations of the ACQ: at baseline and 2 months later (1999).
§ Three administrations of the ACQ: at baseline, 1 week and 6 months later (1998)[17].
¶ r is calculated as Pearson correlation coefficient for frequency, Spearman correlation coefficient for portion size and kappa for container/preparation.
*, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$.

Table 4 Descriptive data and daily median (25th percentile, 75th percentile) citrus food intakes as estimated by the Arizona Citrus Questionnaire (ACQ) administered twice (reproducibility) and compared with the Arizona Food Frequency Questionnaire (AFFQ)

	% Consuming			Grams per day for consumer of product, median (25th percentile, 75th percentile)*		
Citrus group	ACQ1	ACQ2	AFFQ3	ACQ1	ACQ2	AFFQ3
Orange juice	75.8	72.5	78.5	64.3 (12.6, 135.5)	66.0 (13.4, 175.5)	41.6 (12.5, 187.9)
Fresh	5.0	6.7	N/A†			
From frozen concentrate	35.0	30.0	N/A			
Ready made	35.8	35.8	N/A			
Grapefruit juice	40.8	39.2	41.5	12.4 (6.7, 30.3)	16.0 (6.9, 39.9)	13.0 (6.5, 39.0)
Fresh	10.0	15.8	N/A			
From frozen concentrate	9.2	5.0	N/A			
Ready made	21.7	18.4	N/A			
Lemonade	45.8	41.7	3.1	15.3 (8.1, 33.1)	25.4 (12.0, 79.8)	10.6 (5.2, 29.2)
Fresh	12.5	9.2	N/A			
From frozen concentrate	18.3	13.3	N/A			
Ready made	15.0	19.2	N/A			
Orange	77.5	69.2	80.3‡	8.7 (4.4, 46.8)	8.7 (4.4, 18.7)	12.4 (6.9, 44.7)‡
Fresh	75.0	66.7	–			
Canned	2.5	2.5	–			
Grapefruit	65.0	54.0	69.2	17.0 (8.6, 45.8)	16.8 (8.4, 45.4)	15.8 (7.9, 60.1)
Fresh	64.2	53.2	–			
Canned	0.8	0.8	–			
Citrus peel	15.8	15.0	N/A	–	–	–
Marmalade	35.8	35.0	N/A	–	–	–

ACQ1 – first administration of the ACQ; ACQ2 – second administration of the ACQ (after 2-month period); AFFQ3 – Arizona Food Frequency Questionnaire.
* Two-sided sign test comparing intake (g day^{-1}) based on ACQ2 and AFFQ with that based on ACQ1; no statistically significant difference.
† Not applicable.
‡ Includes both orange and tangerine.

Discussion

There is a need for methods to measure long-term dietary intake with adequate accuracy. For large-scale epidemiological studies on chronic diseases, FFQs are often the method of choice to obtain dietary exposure data because short-term recall and diet records methods are generally expensive, unrepresentative of usual intake and inappropriate for assessment of habitual long-term dietary intake. Citrus fruits and citrus peel might not be reported in many single diet records due to large intra-individual day-to-day variation and, therefore, diet records represent an inaccurate estimation of habitual consumption of citrus foods[21]. Most studies reported in the USA and world-wide use regular FFQs to estimate citrus intake in population studies. Moreover, there are no previous data on the use of FFQs in the measurements of D-limonene intake and nationally available nutrient analysis databases do not assess D-limonene.

In this study, the reproducibility of habitual citrus foods and citrus peel intakes estimated by a detailed citrus questionnaire (ACQ) was explored. According to the present results, agreement between the ACQ and AFFQ is good. Moreover, the developed citrus questionnaire provided highly reproducible estimates of citrus foods

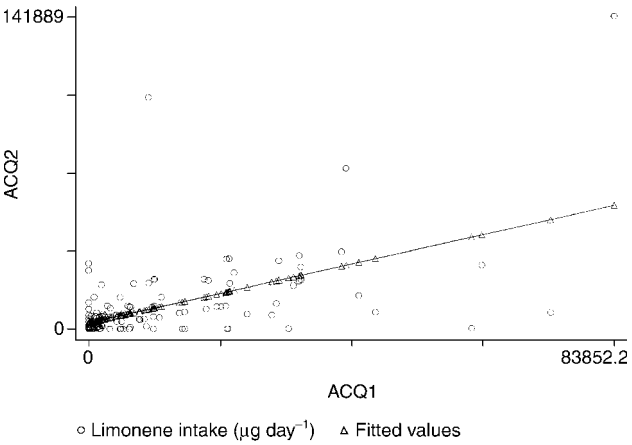

Fig. 1 Daily D-limonene intake (in μg) between two administrations of the Arizona Citrus Questionnaire (ACQ)

Table 5 Daily median (25th percentile, 75th percentile) D-limonene intake (mg day^{-1}) as estimated by the Arizona Citrus Questionnaire (ACQ) administered twice

	Mg/day for consumer of product, median (25th percentile, 75th percentile)*		
Citrus	ACQ1	ACQ2	r†
Total citrus juices	6.7 (2.1, 15.2)	6.6 (1.7, 21.1)	0.74
Orange juice	4.6 (1.0, 10.4)	4.9 (1.0, 13.9)	0.73
Grapefruit juice	0.4 (0.1, 0.7)	0.3 (0.1, 0.6)	0.63
Lemonade	1.1 (0.4, 5.3)	2.5 (0.5, 4.9)	0.58

ACQ1 – first administration of the ACQ; ACQ2 – second administration of the ACQ (after 2-month period).
* Two-sided sign test comparing intake (mg day^{-1}) based on ACQ2 with that based on ACQ1; no statistically significant difference.
† Spearman correlation coefficients.

and peel consumption and D-limonene intake. For epidemiological applications, the capability of a questionnaire to categorise or rank individual subjects by level of their actual intake is generally viewed as its most essential characteristic[22], and is more important than the capacity to measure group means. Determination of associations between D-limonene intake and disease requires accurate ranking of individuals into quantiles of intakes, but not necessarily accurate assessment of absolute intake. This ranking ability can be evaluated by using Spearman's correlation and/or the Wilcoxon signed-rank test. It has also been advocated that to set diet guidelines and to evaluate the magnitude of purported health problems, dietary instruments should estimate absolute intakes. The Pearson's correlation reflects the degree of agreement between the absolute levels of estimates. In the present study, no clear discrepancies were observed between Pearson and Spearman correlations (data not shown). In addition, the two-sided Wilcoxon signed-rank test showed no statistically significant difference between the two dietary methods or the two administrations of the ACQ.

Many studies have examined the reproducibility of nutrient intakes estimated using FFQs. These studies have found correlation coefficients that generally range from 0.5 to 0.8[23–26]. In our study, the Pearson correlation coefficient for D-limonene was 0.6, which falls within the typical range. Moreover, the intra-class correlation was as high as 0.84. On the contrary, fewer studies have examined the reproducibility of intake measurements for specific food items on FFQs. Moderate to high correlation coefficients, in the range of 0.4 to 0.7, have been reported for periods ranging from 1 week to 12 months[27]. In the present study, the ACQ provided highly reproducible estimates of citrus consumption, and the correlation coefficients for citrus foods ranged from 0.48 (citrus peel) to 0.96 (orange juice).

In summary, D-limonene intake may vary not only because the actual amount of citrus consumption differs, but also because the concentration of D-limonene differs by type of juice container and/or preparation. Estimation of the total amount of D-limonene intake in epidemiological studies should include information on the type, preparation and container of the consumed citrus juice. Therefore, we developed the Arizona Citrus Questionnaire (ACQ) to provide an accurate tool for collecting dietary information on citrus consumption, and we then measured its reliability. In this study, estimates of citrus consumption and D-limonene intake from citrus were highly reproducible when the questionnaire was administered at two and three points in time. These data provide additional evidence that a simple, self-administered, semi-quantitative citrus frequency questionnaire can provide useful information on citrus and D-limonene intake. Our approach should provide a useful first tool for assessing the epidemiological association between D-limonene consumption and cancer risk.

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