IS NARINGIN A NATURAL COMPONENT OF LEMON JUICE?

A. Gledhill, D. Roberts, D. A. Hammond

Introduction

Due to the very high acidity of lemon juice, it is not normally consumed as a 100 % product. In Europe it is widely used as a natural acidulant to control the Brix to Acid ratio of 100 % juice products, such as "pineapple juice with a squeeze of lemon", or in juice containing beverages. As part of the European Fruit Juice Association (AIJN) Code of Practice there is a reference guide for lemon juice (1). This details certain critical parameters that a lemon juice on sale in Europe should meet, such as a minimum Brix, maximum levels of regulated heavy metals, hydroxmethylfurfural, and some hygiene parameters. It also contains a range of other parameters, such as the levels of sugars, acids, minerals and isotopic values that are very useful in assessing the quality and authenticity of the juice.

The assessment of authenticity data is a highly complex matter and AIJN recommends that this is left to experts. This is important as the various parameters should not only be assessed individually, i.e. they fall within the specified range but the overall data has to "make sense" for a juice of that particular origin. This information cannot be derived simply from the reference guides and this is where the "expertise" of the expert testing laboratories lies in their assessment of the data.

Most producers are honest and they deliver the product that they are contracted to supply. However, there are always a few that are prepared to supply adulterated products. This is often a particular problem where authenticity checks have not recently been applied. This happened in early 2012 in America with lemon juice which was used for cooking and as a "condiment" for pancakes. Although four of these products were labelled as 100 % lemon juice, analysis clearly showed that they were not pure. The analysis indicated that these particular products had estimated juice contents between 10 and 35 % (2)! This is not an abnormal problem with juices which are not routinely checked.

Although organising an ongoing Quality Assurance program for incoming raw materials is not cheap, it can be a much cheaper option to organising a large scale product recall and all the other ancillary issues involved, such as litigation. This is where the European Quality Control Scheme (EQCS) and Institute of Raw Material quality Assurance (IRMA) systems in Europe help reduce the risk to packers and producers.

"Polyphenols" are not only of interest to marketing departments and consumers for their perceived health benefits, they also provide very useful markers for the juice expert. Most of these compounds absorb in the ultra violet region at 280nm and some years ago Wade's group, at Procter and Gamble, developed an HPLC method to produce characteristic fingerprints for juices (3). The method provides very useful data on a wide range of juices, but due to the complex nature of the chromatograms some profiles are hard to interpret.

The flavonoids are a special class of these compounds, which are very useful for differentiating between different citrus fruits. Before the development of DNA methods (4, 5) the flavonoids and the level and types of carotenoids (6) were used to detect ad-mixtures of citrus juices. Each citrus fruit has a pattern of these compounds which allows you to differentiate between the types (3) and detect the addition of another citrus fruit. Sweet orange *(Citrus sinensis)* for instance has hesperidin and narirutin as two of the major flavonoids, whereas grapefruit *(Citrus paradisi)* contains these two plus an additional compound naringin that is responsible for grapefruit's bitter taste. These patterns allow the addition of small amounts of grapefruit to orange juice to be detected at the ca 0.5 to 1 % level, which is much better than the present DNA methods, where the detection limit (DL) is at least 2 %.

In the AIJN reference guides for citrus fruits (orange, grapefruit, mandarin and lemon) there is a section for the flavonoid compounds that are characteristic of each fruit. For instance for orange a level for hesperidin, its major flavonoid, is quoted in its reference guide, whereas for grapefruit a level is quoted for naringin. The Code of Practice offers two different approaches for flavonoid quantification:

a) the first is the old fashioned colorimetric procedure of Davis, which was published in the 40's (8) and should really no longer be used as it is a non-specific procedure.
b) the second, and more useful procedure, is an HPLC method validated by the IFU analytical commission that allows the flavonoid compounds to be separated and quantified by UV detection (9).

In Rouseff's chapter in the "white" Authenticity Book (10) he proposed a further HPLC procedure that could differentiate between many of the different citrus fruits. Unlike in Europe, in the US (12) and under the Codex standard for fruit juices (13) it is permitted to add up to 10 % tangerine/mandarin juice (Citrus reticulata) to orange juice without the need to label the product as a mixed juice. This addition would of course also be possible in Europe, but here the product would have to be labelled as a mixed juice e.g. "orange and tangerine juice". During the recent discussions about the new European Fruit Juice directive (14) some countries pushed for this interpretation to be allowed under EU law. However, in the final form of the Regulation this was not permitted.

Lemon contains a different pattern of flavonoids to orange and grapefruit, with eriocitrin and hesperidin being the major compounds (11). In this extensive review of the literature of the flavonoids seen in Citrus fruits it stipulates that a low level of naringin ca 1 mg/l can be seen in lemon juices. In the AIJN reference guide it quotes a value for hesperidin by the Davis method, as this procedure cannot differentiate between the various flavonoids and a value by HPLC. It also mentions eriocitrin as the characteristic flavonoid for lemon and lime. In 2010 the AIJN expert group changed the wording of the reference guide for naringin, the old wording stated that a peak could be seen in the chromatogram near to naringin, whereas the new wording stated that naringin was not a normal component of lemon, which was the consensus of the expert group. This new wording led to problems for some producers when a peak arose in the HPLC chromatogram in the region where naringin eluted in their juices using the IFU procedure # 58. Even using HPLC linked with diode array detection the compound in question returned a UV spectrum which was similar enough to be classified as naringin by the chromatographic software.

It was decided to investigate this further to determine whether this compound was actually naringin, and the AIJN experts were incorrect. To undertake this study it was decided to use the Kirksey HPLC procedure (3) for polyphenols as it has a longer analysis time than the IFU method and could possibly give better resolution of the compounds in question. The method was modified to use a column with a smaller particle size, which significantly enhances the resolution and reduces the analysis time from 65 minutes to under 20 minutes.

SAMPLES

The samples examined in this study were a mixture of clear and clarified lemon juice concentrates from Argentina, UK retail lemon juices, a lemon squash, lemon juice pressed in the laboratory and two grapefruit juices, one clear and one clarified. These are detailed in Table 1.

TAB. 1: SAMPLES USED IN LC-MS STUDY

Sample #	Juice type	Origin
1	Cloudy lemon juice concentrate A	Argentina
2	Cloudy lemon juice concentrate B	Argentina
3	Cloudy lemon juice concentrate C	Argentina
4	Cloudy lemon juice concentrate D	Argentina
5	Cloudy lemon juice concentrate E	Argentina
6	Cloudy lemon juice concentrate F	Argentina
7	Clear Lemon juice concentrate A	Argentina
8	Clear Lemon juice concentrate C	Argentina
9	Clear Lemon juice concentrate D	Argentina
10	Clear Grapefruit juice concentrate B	Argentina
11	Grapefruit Juice Tropicana	Unknown
12	Co-op lemon juice from concentrate	Italy & Argentina
13	Co-op whole lemon Squash	unknown
14	Lemon juice from concentrate BB Nov 12	unknown
15	Lemon juice from concentrate BB Dec 12	unknown
16	Fresh lemon pressed in the laboratory (5/4/12 sulfite preserved)	unknown
17	Fresh lemon pressed in the laboratory 7/5/12	unknown

Cloudy and clarified lemon juice concentrates were chosen for this investigation to determine if there was any effect due to the clarification step. A lemon squash was chosen as this contained a comminuted lemon base and so may indicate if this problem was associated with the non-edible portions of the fruit (flavedo and/or albedo). Two juices pressed from lemons were taken one was pressed in our Juice authenticity laboratory in the US and had been shown to contain the "suspect" peak. Sodium metabisulfite was added to this material and it was dispatched to the UK for analysis. Two grapefruit juices were included for reference purposes. The juice concentrates were diluted to single strength in laboratory grade water. All the products, at single strength, were subject to a high speed centrifugation and filtration prior to analysis by Ultra Performance Liquid Chromatography-diode array detection (UPLC-DAD) linked in series with a Time Of Flight (TOF) mass detector for peak detection and enhanced characterisation.

ANALYSIS METHOD

The analysis was carried out on a Waters ACQUITY I-Class UPLC(™) which contained a diode array detector, set to collect data from 190 to 600 nm. The eluant from this detector was then fed into a Waters Xevo G2 Qtof(™) mass spectrometer (MS). The MS and UPLC(™) conditions are given in Tables 2 and 3 respectively.

TAB. 2: MASS SPECTROMETER CONDITIONS USED IN THIS STUDY	
Ionization Mode:	ESI-ve at 0.7 kV
Cone voltage:	25V
Desolvation Temperature:	450˚C
Acquisition Range:	50-1200 m/z
Acquisition Rate:	10 spectra/second
Collision Energy Ramp:	25-35eV
Resolution:	22000 FWHM

TAB. 3: UPLC(™) CONDITIONS		
Column:	Waters ACQUITY UPLC(™) BEH C18	
Column & Particle sizes	150 mm x 2.1 mm, 1.7 µm	
Column temperature:	45ºC	
Flow rate :	0.45 ml/min	
Mobile phase A =	10mM Ammonium acetate in HPLC H2O	
Mobile phase B =	10mM Ammonium acetate in HPLC grade MeOH	
Injection Volume:	5µL	
Gradient:	A (%)	B (%)
Time(min)	99	1
Initial	99	1
0.25	70	30
10.1	1	99
10.2	1	99
12	99	1
12.1	99	1
15	99	1

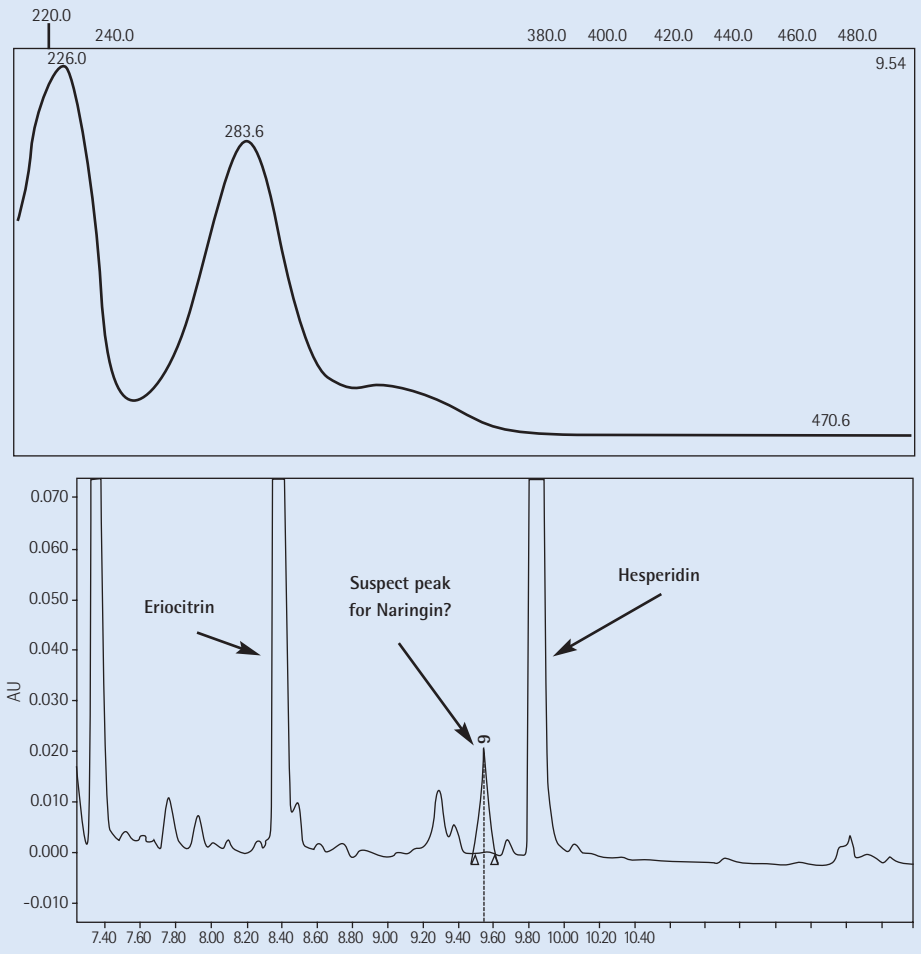

Fig. 1: UPLC trace run on an H class Waters ACQUITY system showing suspect naringin peak with its UV spectrum

DISCUSSION

The original HPLC method of Kirksey used a 15 cm C_{18} column with a small particle size (3µm) but it was found that a regular 25 cm C_{18} column with 5 m particles worked as well. These column configurations gave an analysis time of 65 minutes which is quite long for a single analysis. However, switching to UPLC(TM) conditions allowed the analysis to be conducted in about one quarter of the time without a significant loss of resolution. Shown in Figure 1 is a typical chromatogram of lemon juice showing the suspect naringin peak.

The Water's software identified this peak as naringin and examination of the spectral shape also showed a very close visual match to naringin. This chromatogram was run using a Waters "H" class ACQUITY UPLC(TM) system. The phosphate buffer given in the "short" Kirksey gradient was replaced with 0.6 % acetic acid as it was "easier" on the pump seals. When the method was transferred onto MS analysis the solvents were again changed from acetic acid to an ammonium acetate buffer and methanol replaced the acetonitrile as the organic modifier. The UPLC(TM) system was also changed to an "I" class ACQUITY.

These modifications lead to slightly shorter retention times for naringin and hesperidin but similar resolutions. Six of the lemon juice concentrates, two of the retail lemon juice from concentrate products and one of the laboratory pressed juices showed peaks in the UV chromatograms where naringin eluted and also had a spectrum similar to naringin, which would mean that they could be incorrectly assigned as containing naringin (table 4).

The majority of the cloudy lemon juice concentrates showed this unusual peak, whereas the majority of the clarified lemon products were clean. Although this might suggest that the problem was only present in the cloudy products, as one of the cloudy ones was clean and the peak was seen in one of the clarified juices, this suggests that the clarification process was not relevant to the issue.

However, analysis of these products using mass spectral detection showed a very different picture. Scanning the chromatograms using a mass fragment ion of m/z 579.17, the molecular ion (MI) of naringin, showed that there were no peaks in the region where naringin elutes in the concentrate samples, as illustrated in the uppermost chromatogram in Figure 2. This clearly showed that

TAB. 4: UV AND MS ASSESSMENT OF UPLC CHROMATOGRAMS FOR SAMPLES

Sample No	Type	Origin	UV detection 283 nm	MS detection (m/z)
1	Cloudy lemon juice concentrate A	Argentina	negative	NA
2	Cloudy lemon juice concentrate B	Argentina	positive	609.18
3	Cloudy lemon juice concentrate C	Argentina	positive	609.18
4	Cloudy lemon juice concentrate D	Argentina	positive	609.18
5	Cloudy lemon juice concentrate E	Argentina	positive	609.18
6	Cloudy lemon juice concentrate F	Argentina	positive	609.18
7	Clear lemon juice concentrate A	Argentina	positive	609.18
8	Clear lemon juice concentrate C	Argentina	negative	NA
9	Clear lemon juice concentrate D	Argentina	negative	NA
10	Clear grapefruit juice concentrate B	Argentina	positive	579.17
11	Tropicana grapefruit Juice	Unknown	positive	579.17
12	Co-op lemon juice from concentrate	Italy & Argentina	negative	NA
13	Co-op whole lemon Squash	Unknown	negative	NA
14	Lemon juice from concentrate BB Nov 12	Unknown	positive	579.17
15	Lemon juice from concentrate BB Dec 12	Unknown	positive	579.17
16	Fresh lemon pressed in the laboratory (5/4/12 sulfite preserved)	Unknown	positive	609.18
17	Fresh lemon pressed in the laboratory 7/5/12	Unknown	negative	NA

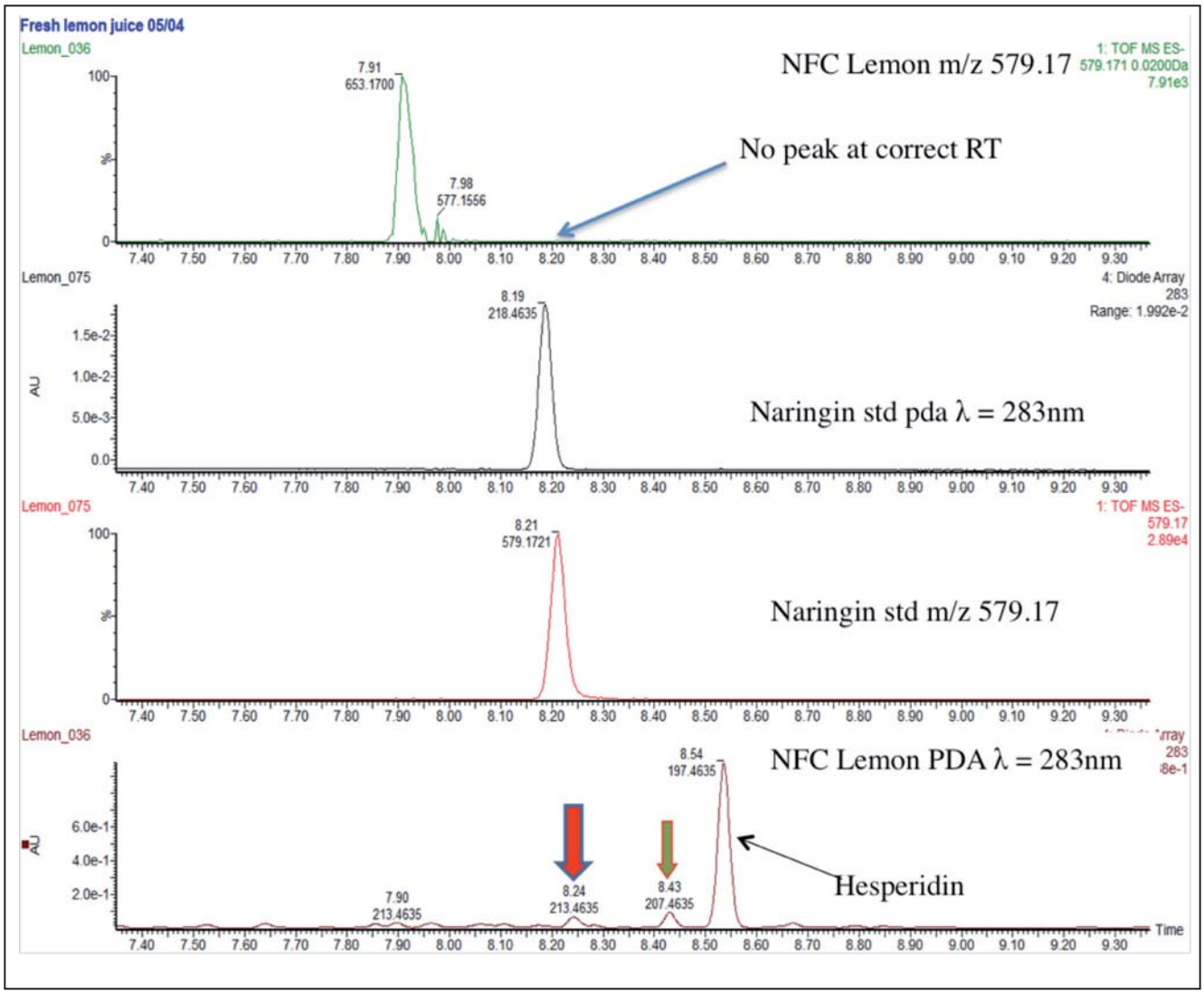

Figure 2: Diode array and MS chromatograms for lemon sample with suspect peak and naringin standard

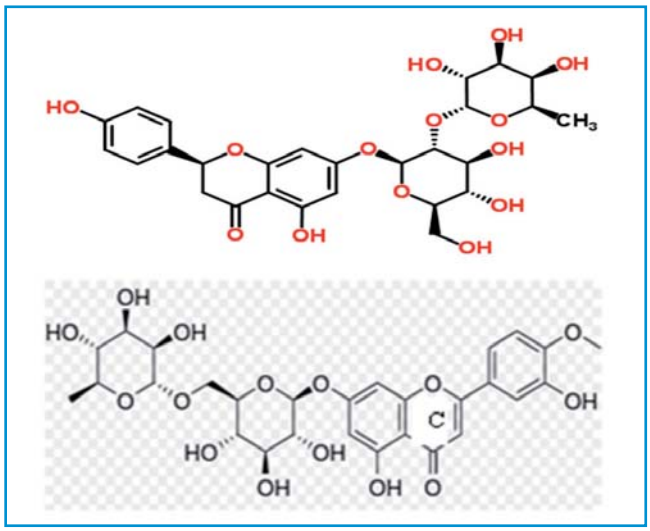

Fig. 3: Chemical structure for naringin (left) & diosmin (right)

the samples do not contain any naringin and this peak was actually due to another compound with a similar UV spectrum to naringin. The next two chromatograms in this figure show the peaks for a naringin standard using DAD and MS detection. The final chromatogram shows the UV chromatogram for a lemon sample with the suspect peak highlighted by the red arrow. The second large arrow highlights another peak in this chromatogram, which if conventional HPLC had been used with its lower peak resolution, may have been confused with naringin to. However, in this case the UV spectrum is slightly different. Accurate MS analysis indicated that this second peak was diosmin, which has a very similar structure to hesperidin except that the oxygen hetrocyclic ring, marked C in figure 3, is further oxidised to give a more conjugated system and hence slightly different UV spectrum, not shown here.

Mass spectral analysis of the samples showed that the suspect peak in the products showed a molecular ion of m/z 609.18, which is also the accurate mass for hesperidin. However, the different elution time indicates that this is not actually hesperidin. The fragmentation pattern of the MI shows peaks at 299 and 301 m/z which are also seen in hesperidin and diosmin. This indicates that the basic structure of this compound is hesperidin in nature but from the available information the actual structure of the compound could not be identified. Due to the very similar structural similarities between hesperidin and naringin this explains why DAD spectra were very similar. No suspect peak was identified in the lemon squash sample which would suggest that the peak is not related to the albedo or flavedo portions of the fruit as if this was the case a higher level would have been seen in this product as it had been prepared from a comminuted base e.g. from mincing/pressing the whole fruit.

The regular and clarified grapefruit juices, as expected, showed peaks for naringin in the UV and MS chromato-

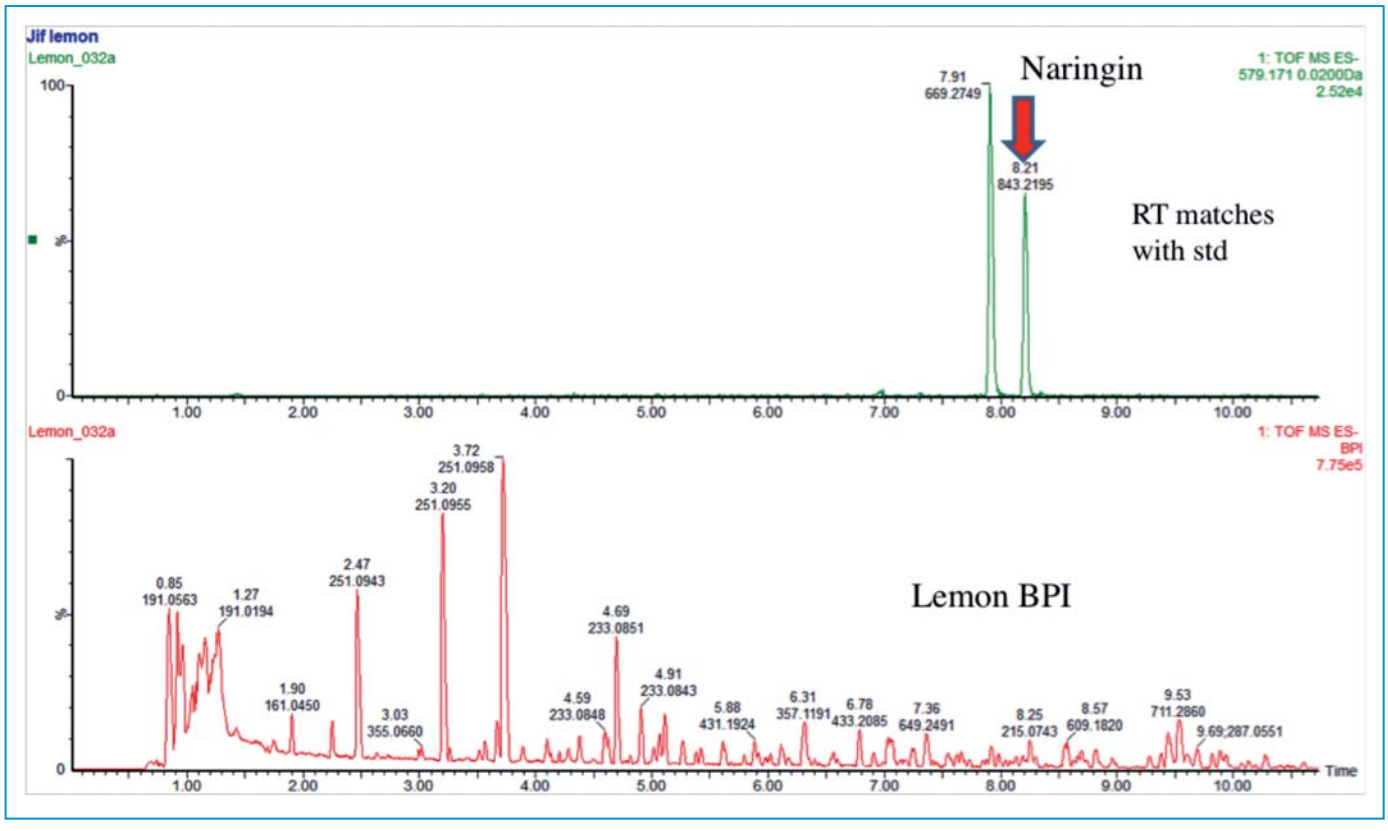

Fig. 4: Extracted ion and BPI chromatograms for lemon sample 14

grams. However, in two of the UK retail lemon samples 14 & 15 a peak was seen with the same retention time as naringin, had the correct MI at 579.17 m/z and its spectrum also matched the standard (not shown here), Figure 4. It showed also be considered that these two products were aimed at cooking applications and may well show similar problems of adulteration in the UK to those seen earlier in 2012 in the US for this type of product.

CONCLUSIONS

Mass spectral analysis, accurate mass and fragmentation pattern, showed that naringin is not a normal component of Argentinean lemon juice. However, there can be a peak with a very similar retention time to naringin, which is very difficult to exclude as naringin from its DAD spectrum. In the lemon juice concentrates and the NFC lemon juice that showed the suspect "naringin" peak, no peak was evident in the chromatograms when naringin's accurate mass was selected.

The accurate mass for the suspect peak identified the molecular formula of this compound to be C28H34O15, which is the same formula as hesperidin. However, hesperidin has a slightly longer retention time under these analysis conditions. The ion fragment at m/z 301 also supports the identification of a hesperidin "type" compound. To complicate the story there is also a peak in the spectrum at 607 m/z which would relate to C28H32O15 (diosmin) and the spectrum also shows a fragment at 299 m/z. Although the analysis has clearly demonstrated that the suspect peak is not naringin, its structure could not be fully indentified from the accurate mass and fragmentation data.

Two retail lemon juice samples sold for cooking applications were found to contain naringin. This was easily detected and confirmed using mass spectrometry and indicated that the lemon juices were not 100 % and had been mixed with another juice (grapefruit or sour orange).

This study also showed a significantly reduced analysis time by using the UPLC(TM) configuration over conventional HPLC. It also illustrates the potential that the combination of UPLC(TM) and mass spectrometry offers in the detection and conformation of fruit juice adulteration.

References

(1) Anonymous, reference guide for lemon juice. AIJN website www.AIJN.org
(2) National Consumer league website: http://nclnet.org/food/85-food-labeling/637-watered-down-lemon-juice-making-advocates-sour
(3) Kirksey et al (1995). In: Methods to detect adulteration of fruit juice beverages Vol. 1, Pages 145 – 165, Published by AgScience, Auburndale Fl
(4) Knights (2000). Agro Food Industry Hi-Tech Vol. 11 (2), 7 - 8.
(5) Moreau & Canivenc (2008). Fruit Processing May/June 156 - 159
(6) Anonymous, reference guide for orange juice. AIJN website WWW.AIJN.org
(7) Anonymous IFU method 59. IFU website www.IFU-fruitjuice.com
(8) Davis (1947). Ind. Engng. Chem. 19, 476
(9) Anonymous IFU method 58. IFU website www.IFU-fruitjuice.com
(10) Rouseff (1990) Chapter (X) In: Adulteration of Fruit Juice Beverages, Ed by Nagy, Attaway & Rhodes. Published by Marcel Dekker Inc.
(11) Peterson et al (2006). J. of Food Comp. & Anal 19, 574 - 580
(12) FDA website 21 CFR 146.146 http://www.accessdata.fda.gov/scripts/cdrh/ cfdocs/cfcfr/CFRSearch.cfm?fr=146.146&SearchTerm=orange%20concentrate
(13) Codex Standard for Fruit Juices and Nectars, 2005, 247 http://www.codexalimentarius.org/standards/list-of-standards/en/?no_cache=1
(14) New European Fruit Juice Directive EU 2012/12 http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:115:0001:0011:EN:PDF

AUTHORS

A. Gledhill, D. Roberts; Waters Corporation, Manchester
UK-M23 9LZ Manchester – United Kingdom

D. A. Hammond; Eurofins Scientific
F-44323 Nantes – France

www.waters.com / www.eurofins.com