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A Rapid Liquid Chromatography Electrospray Ionization Mass Spectrometry^n Method for Evaluation of Synephrine in *Citrus aurantium* L. Samples

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Immature bitter orange fruit and its extracts have been introduced into the market as an alternative to Ephedra in weight loss products. However, the safety of the immature bitter orange fruit and its extracts is a debated argument due to the presence of synephrine, a constituent known as a sympathomimetic agent. In this paper, we describe the development of a new, rapid, and simple liquid chromatography−electrospray ionization−tandem mass spectrometry method devoted to the quantitative determination of synephrine in bitter orange samples, containing a high quantity of synephrine, and sweet orange samples, known to contain a low level of synephrine but at the same time being one of the main synephrine sources in a normal human diet. Two bitter orange dry extracts containing 5 and 6% sSynephrine and 10 sweet orange samples have been analyzed. Between the sweet orange samples, six were fresh oranges and four were fresh-squeezed juices; in these samples, the synephrine levels ranged from 0.00128 to 0.00349%.

KEYWORDS: ***Citrus aurantium* L. var. amara (L.); *Citrus aurantium* L. var. sinensis; bitter orange; bitter orange extract; sweet orange; sweet orange juice; synephrine; LC-ESI-MS/MS method**

INTRODUCTION

Bitter orange trees exhibit botanical characteristics similar to those of sweet orange, and the *Citrus* taxonomy is complex due to the high degree of hybridization that has taken place. There are three schools of thought regarding it, one being that there are indeed only three species with all varieties being subspecies within these three species. In the more traditional taxonomic scheme, recognized by Swingle, sweet orange is classified as *Citrus sinensis* (L.) Osbeck, as a separate species (*1*, *2*), but in a more recent classification, according to Penso, bitter and sweet oranges are regarded as subspecies or varieties of *Citrus aurantium* L. (Rutaceae). These are named, respectively, *C. aurantium* L. var. *amara* (L.) and *C. aurantium* L. var. *sinensis* (L.) (*3*).

The bitter orange is not widely cultivated as the sweet one. The Mediterranean main producers are Spain (Seville and Malaga), Italy (Sicily), Libia (Tripoli), and Malta. The bitter orange tree appears to have been introduced from northern India into eastern Africa, Arabia, and Syria, whence either Arabs or the Crusades brought it to Europe about AD 1200. The sweet orange, as well as the bitter one, appears to be of Chinese origin (*4*, *5*).

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Many parts of the bitter orange tree as well as of the sweet orange are currently used in a wide number of phytotherapeutic treatments. Bitter orange peel and its alcoholic tincture, bitter and sweet essential oils, and sweet orange flowers are official in European Pharmacopoeia (EP 5.0, 2005), while sweet orange oil and sweet orange peel tincture are official in the United States National Formulary (NF 23, 2005).

Bitter and sweet orange peels exhibit numerous and distinct pharmacological activities, including antiinflammatory, antibacterial, and antifungal ones (*5*). Sweet orange fruit is the most used citrus in human feeding, exhibiting an agreeable taste and having a high nutritional value.

The dried, unripe bitter orange fruit (Zhi-Shi) in traditional Chinese medicine is used primarily to treat digestive problems: indigestion, abdominal distension, dysentery, or constipation. The contemporary use in Chinese medicine is, as an intravenous infusion, in the treatment of toxic and anaphylactic shock as well as for weak heart conditions and cardiac exhaustion (*6*).

Nowadays, in Western countries, a wide interest around the unripe fruits of bitter orange is grown due to its use in herbal weight loss products. Immature dried bitter orange fruit contains up to 10% of flavonoids and a number of phenyl-ethyl-amines including *N*-methyltyramine, octopamine, and above all synephrine (0.2%) (*6*). For its pharmacological interest, synephrine (**Figure 1**) is, also, a synthetic drug developed as a sympathomimetic agent (Boehringer Ing. German Patent 566.578, 1931),

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exhibiting various pharmacological activities such as vasoconstriction and bronchial muscle relaxation. Its presence in the peel of oranges (*7*) and in the immature fruits of bitter oranges (*6*) as well as in sweet orange juice (*8*, *9*) has been proved.

Immature bitter orange fruits, as a consequence of its amine content, might produce effects on human metabolism, which could be useful for reducing fat mass in obese humans. All of the alkaloids, and synephrine above all, are adrenergic agents that raise metabolic rates and oxidation of fats through an increased thermogenesis and stimulate lipolysis presumably by means of β-3-receptors. These receptor subtypes appear to be responsible for the lypolytic and thermogenic effects of adrenergic agents, while subtypes β-1 and β-2 control cardiac effects. It must be noticed that an ideal fat-losing agent should have a preponderance of β-3 interactions and according to some researchers, the alkaloids present in bitter orange seem to satisfy this condition (*10*).

Recently, there was an increasing concern in the health risks associated with the consumption of immature bitter orange fruit extracts, for their possible effects in raising blood pressure and activating adverse cardiac effects (*11*). There is, indeed, evidence that bitter orange fruits can produce cardiac disturbance in animals. Repeated oral administrations in rats of two different bitter orange unripe fruit extracts (containing 4 and 6% synephrine) significantly produced slimming effects, reducing food intake and body weight gain, without modification of arterial blood pressure but with significant alterations of ECG parameters (ventricular arrhythmias with enlargement of the QRS complex) (*12*). In hypertensive rats (after partial portal vein ligation), the bitter orange fruit water extract, infused via a syringe pump, reduced the portal pressure, possibly by way of arterial vasoconstriction (*13*).

In humans, pure synephrine, when administered by continuous intravenous infusion at the rate of 4 mg/min, raised significantly systolic and mean arterial pressures, whereas diastolic pressure and heart rate were unchanged (*14*). However, it must be emphasized that the results of this study are not directly applicable to oral consumption of bitter orange extracts in dietary supplements, either for the administration way (intravenous) or for the utilization of pure synephrine (as noted by AHPA, News in September 2004) (*15*).

Indeed, further studies demonstrated the safety of oral administration of bitter orange fruit or synephrine. In normotensive humans, the ingestion of freshly squeezed Seville orange juice, corresponding approximately to 13−14 mg of synephrine, did not raise blood pressure or change significantly the hemodynamic measurements (heart rate, systolic, diastolic, and mean arterial pressures) (*16*). In a double-masked, randomized, placebo-controlled study of bitter orange extract (975 mg), an association with caffeine and St. John's Wort did not produce significant change in blood pressure, heart rate, electrocardiographics findings, serum chemistry, or urinalysis in overweight healthy adults (*17*).

However, the safety of the immature bitter orange fruit and its extracts is still a debated argument. Currently, immature bitter orange fruits and their derivatives have a different regulatory status. In the United States, bitter orange is usually employed as a dietary supplement, and its dry extracts, standardized to 6% of synephrine, are widely used in weight loss products. In Canada, it is an approved active ingredient in some over the counter schedules, and it is considered a traditional herbal medicine requiring premarketing authorization. In the United Kingdom, it is an herbal medicine entered in the General Sale List, table A (internal or external use) of Schedule 1 and requires a full Products License.

In Italy, the immature bitter orange fruit can be used as an ingredient of dietary supplements. However, the Ministry of Health has established a limit of 30 mg daily for the maximum consumption of synephrine, corresponding to 800 mg of a dry extract standardized to 4% synephrine. The herbal dietary supplement's label has to report the synephrine content at the advised dosage and must be approved by the Ministry of Health.

Looking at the above-cited pharmacological studies as well as the regulatory requirements, the development of analytical methods devoted to the quantitative determination of synephrine in *C. aurantium* L.-based products is surely of high interest.

For this aim, high-performance liquid chromatography (HPLC) methods with electrochemical detection (*18*), fluorescence detection (*19*), ultraviolet (*20*, *21*), or photodiode array detection (*19*, *22*, *23*) (DAD) have been proposed in the literature, and the last two are usually employed in quality control of phytochemical products. The cited papers deal with bitter orange samples. Only Kusu in 1996, in the frame of his study on synephrine's enantiomers separation, analyzed a sample of sweet orange, from the Valencia cultivar (*18*).

Considering the complexity of the natural extracts, the development of a liquid chromatgraphy $(LC/MS)^n$ method seemed to be of particular interest due to its higher specificity. In this context, Gay et al. in 2003, during the 9th Annual FDA Science Forum, reported the first LC/tandem mass spectrometry (MS/MS) method for synephrine determination in dietary supplements containing bitter orange (*24*). The results were obtained in atmospheric pressure chemical ionization (APCI) conditions.

In this paper, we describe the development of a new LC-electrospray ionization (ESI)/MS/MS method for the synephrine analysis in *C. aurantium* L. var. *amara* (L.) and *C. aurantium* L. var. *sinensis* (L.) samples. The method is rapid, simple, and could be suitable for the quantitative determination of synephrine in fresh and dried fruits and dried extracts of various *C. aurantium* L. samples. Its application for tarocco, navel, and naveline oranges and four samples of sweet orange juices stored at +4 °C, directly bought from the market, is discussed.

MATERIALS AND METHODS

Materials. Synephrine (purity 99.9%, HPLC grade) was purchased from Sigma Aldrich (Italy). Acetonitrile HPLC grade was purchased from BDH Laboratory Supplies (Poole, England), Formic Acid HPLC grade was purchased from Merck (Darmstadt, Germany). Syringe filters were purchased from GE Osmonic Labstore (Minnetonka, MN). Water was purified using a Modupure Plus model LBMPP 20 1200 (Continental Water Systems Corporation, United States).

The samples analyzed were as follows: *C. aurantium* L. var. *amara* (L.) unripe fruit standardized dried extract with 6.0% synephrine content, obtained from an Italian supplier (Advantra Z, Sochim International SpA), and an unripe fruit freeze-dried extract with 5.0% synephrine content produced by Aboca. These samples were used as standards and diluted 1:10000 in order to obtain analytical samples with synephrine content analogous to that present in orange pulp and orange juice samples. *C. aurantium* L. var. *sinensis* (L.) oranges, tarocco, naveline and navel, as well as fresh orange juices stored a +4 °C, were directly acquired from the market.

Instrumentation. LC analysis were performed using an Agilent Technologies (Palo Alto, CA) modular 1100 system consisting of a vacuum degasser, a binary pump, a Peltier thermostated autosampler, a Peltier thermostated column compartment, and an ion trap mass selective detector. A Prodigy RP-18 (4.6 mm × 250 mm, 100 Å, 5 μm, Phenomenex, United States) analytical column protected by a RP-18 security guard cartridge (4 mm × 3 mm) was used.

Table 1. Operative Parameters Employed for (**a**) MS and (**b**) MS/MS Measurements

Section a

nebulizer pressure (psi)	60	Oct. RF (Vpp)	122.9
dry gas flow (L/min)	11	lens 1 (V)	–5
dry gas temperature (°C)	350	lens 2 (V)	–60
capillary (V)	–3000	ICC	ON
skimmer (V)	40.0	target	30000
cap. exit (V)	103.5	max acc. time (ms)	100
Oct 1DC (V)	12.00	scan (*m/z*)	80–200
Oct 2DC (V)	1.70	average	7
trap drive	43.00	rolling average no.	2

Section b

isolation		MS/MS conditions	
m/z value	width	cutoff	amplitude
168.3	2.0	44	1.00
150.1	1.0		

LC Analysis. The mobile phase was composed of water–acetonitrile–formic acid 64.9:35:0.1 and pumped at 0.8 mL/min in isocratic conditions at room temperature during 10 min, and then, a 1 min column washing with water–acetonitrile 5:95 was performed. The autosampler was thermostated at 20 °C. The column compartment was thermostated at 25 °C.

A standard synephrine solution was directly infused in the ESI source, where positive ions were formed. Mass selective detector operative parameters are reported in **Table 1**.

Plant Extract. The unripe dried fruit of *C. aurantium* L. var. *amara* (L.), purchased from China (Tianjin port free trade zone supplier), was ground and then extracted with ethanol:water 20:80 (v:v). At the end of extraction, ethanol was evaporated under vacuum and the resulting mixture was freeze-dried yielding the correspondent extract (15% of the starting weight).

Samples Preparation. The commercial oranges were peeled and then treated with a juice extractor (Moulinex, France) to separate pulp from the juice. The orange juices and pulps prepared in the laboratory (from tarocco, naveline, and navel oranges) and orange juices bought on the market were treated as follows: 1 g of each sample was treated with 9 mL of the mobile phase (water–acetonitrile–formic acid 64.9:35:0.1) for 1 h, at 30 °C under ultrasonic treatment. The resulting mixture was centrifuged, filtered into a volumetric flask, and brought to the final volume of 10 mL with the same solvent mixture. All of the extracts were filtered through 0.45 μm Cellulose acetate syringe filter (Cameo, 30 mm) before HPLC analysis.

The samples Advantra Z and Aboca's freeze-dried extract were treated as follows: 0.5 g of sample was treated with 100 mL of the mobile phase at 90 °C for 15 min. The mixture was filtered into a volumetric flask and brought to the final volume of 100 mL with the same solvent mixture. The resulting solution was further diluted 1:50 and filtered through 0.45 μm Cellulose acetate filter before introduction in the LC.

Standard Preparation. The stock solution was prepared by dissolving synephrine (10 mg) in the LC mobile phase to achieve the final concentration of 0.5 mg/mL.

Calibration Curves. The stock solution was diluted to obtain samples with synephrine concentration ranging from 0.2 to 6.25 μg/mL. Three 5 μL injections of each concentration were made. The calibration curve was obtained by plotting the mean peak areas vs the corresponding concentrations. The same solution was used to verify the response linearity in orange pulp and juice matrices, by adding to them known amounts of synephrine.

Recovery. The recovery was evaluated by means of the standard addition method. The *C. aurantium* L. sample was spiked by adding a known volume of standard synephrine solutions. The so obtained spiked samples were extracted and analyzed by LC/MS/MS. The synephrine-related peak areas of the spiked samples were plotted vs the synephrine spiked amount, and the original sample concentration was determined by the *x*-intercept from the extrapolation of the experimental curve.

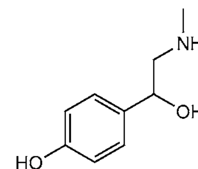


Figure 1. Synephrine structure.

RESULTS AND DISCUSSION

Method Development. The determination of synephrine levels in *C. aurantium* L. and in products containing *C. aurantium* L. is currently carried out by LC/UV or LC/DAD analysis. In our quality control laboratories, by using the LC/DAD method described by Pellati (*22*) with only minor modification, chromatograms of *C. aurantium* L. samples, as those reported in **Figure 2**, are usually obtained. The synephrine peak is detected at $R_t = 15 \pm 0.2$ min.

The limit of this approach is, aside the long analysis time, that synephrine can be identified only by retention time (R_t) and by its UV spectrum. It is obvious that coelution of other analytes, with the same UV spectra in the same R_t window, might lead to misleading results.

It could be noted that LC/MS equipment is much more expensive than an LC/DAD or simply an LC/UV, but as it will be shown, the high specificity and selectivity of the method allow the development of faster analytical procedures. Consequently, the use of mass spectrometry as detection system is surely of interest, in particular when MS/MS experiments can be performed. The development of a LC-MS/MS method would lead to a higher specificity in synephrine quantitative determination.

The preliminary investigation based on APCI led to interesting results. However, considering the low energy deposition present in the electrospray condition, as well as the wider diffusion of this ionization method, it was thought of interest to verify the capabilities of LC-ESI-MS^n approach in synephrine analysis. The final aim was not only to develop a method similar to the APCI-based one or to give a detection method alternative to the well-established analytical approaches but, due to the high specificity of the LC-ESI-MS^n approach, to obtain a significative reduction of the analysis time. First of all, to reach this aim, a preliminary study on the behavior under ESI and collisional conditions (MS/MS) of synephrine was performed.

A methanol solution of pure synephrine was infused by means of a syringe pump into the ESI source. The ESI spectrum mainly shows the presence of protonated synephrine molecules at *m/z* 168 (**Figure 3a**) with a less abundant ion at *m/z* 150, due to primary water loss. In **Figure 3b–d**, the MS^n spectra are also reported, which leads to the fragmentation pattern shown in **Scheme 1**: The protonated molecules show collisional-induced water loss only, leading to the ion at *m/z* 150. This ionic species undergoes a series of decomposition pathways, mainly related to cleavages in the α position to the amino group. Thus, aside a minor $CH_3^\bullet$ radical loss, leading to the ion at *m/z* 135, the two main fragmentation products are detected at *m/z* 119 and 121, the former due to methyl-amine loss and the latter originating by loss of $NHCH_2$. It must be emphasized that the methyl radical loss is an exception of the even electron rule, which states that a radical loss from a cation is a thermodynamically disfavored process (*25*). The formation of the ion at *m/z* 135 can be justified by the high conjugation present in its structure, which easily delocalizes the unpaired electron. Finally, the ion at *m/z* 119 shows a CO loss only, typical of phenolic moieties, and leads to the ion at *m/z* 91, reasonably of tropilium structure.

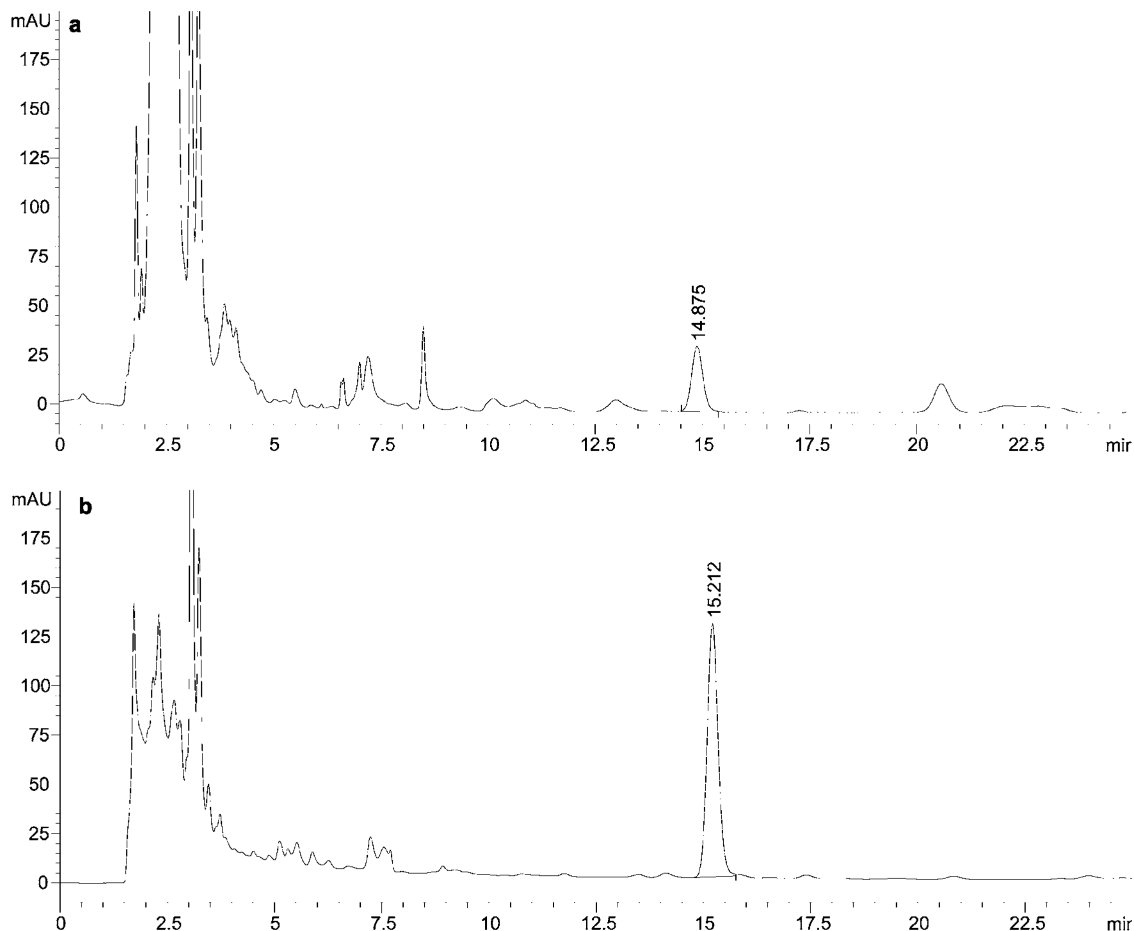


Figure 2. LC/DAD UV chromatograms taken at 220 nm of (**A**) a commercial sweet orange [(*C. aurantium* L. var. *sinensis* (L.)] juice extract (sample 7) and (**B**) Aboca's bitter orange [(*C. aurantium* L. var. *amara* (L.)] freeze-dried extract. The peak at $R_t = 15 \pm 0.2$ min corresponds to synephrine. The chromatograms were obtained using a mobile phase composed of water, containing 0.5% sodium dodecyl sulfate (SDS) and 0.1% orthophosphoric acid (solvent A) and acetonitrile (solvent B). The eluent was composed of solvents A and B in a 65/35 ratio. The elution was performed in isocratic conditions, during 26 min, at the flow rate of 1 mL/min. Then, a column wash was performed using water/acetonitrile in a 50/50 ratio. A photodiode array detector set at 220 nm was used. A Phenomenex prodigy C18 column 250 mm × 4.6 mm, equipped with a security guard was used.

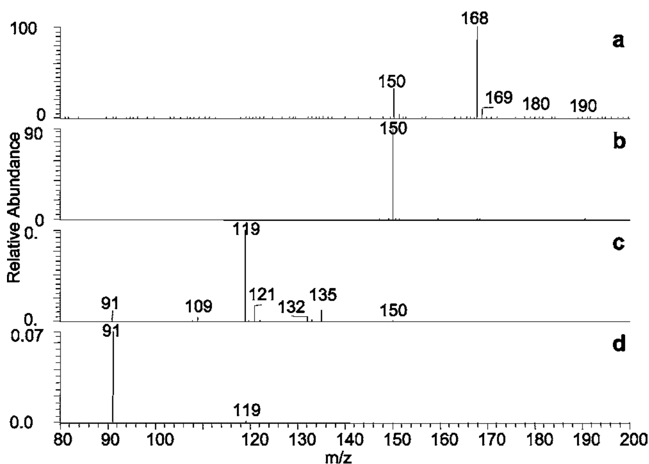


Figure 3. (**a**) ESI-MS spectrum of a standard sample of synephrine. (**b**) ESI-MS^2 ion spectrum of the ion at *m/z* 168. (**c**) ESI-MS^3 spectrum of the ion at *m/z* 150. (**d**) ESI-MS^4 spectrum of the ion at *m/z* 119.

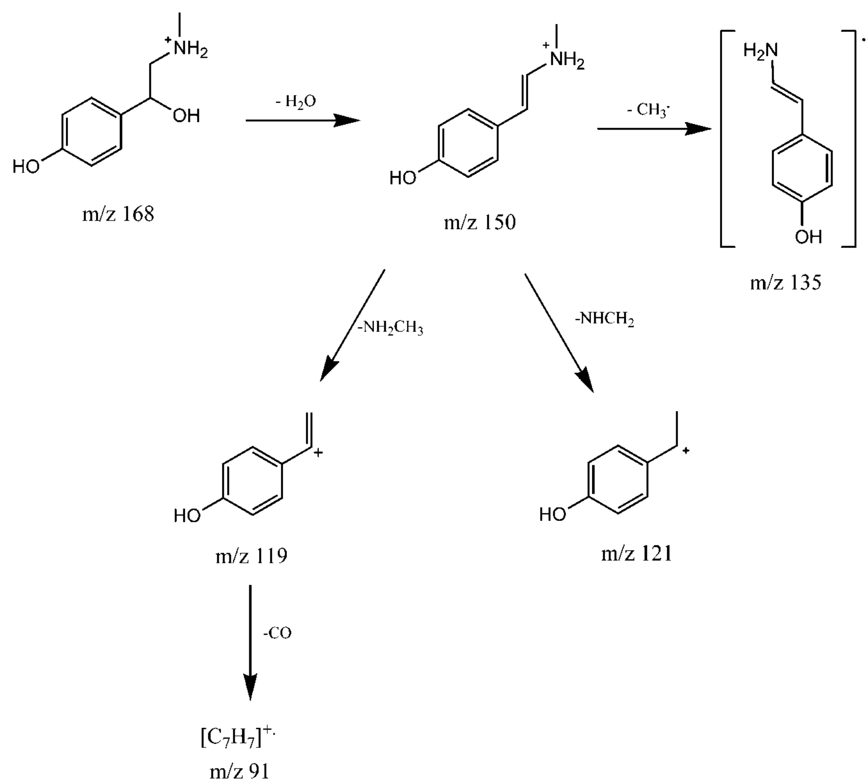
Looking at these data, both the ion due to a protonated molecule at *m/z* 168 and the daughter ion at *m/z* 150 were chosen for a specific and selective synephrine determination.

The analytical LC conditions already reported in the literature for synephrine quantitative determination by means of DAD detector were not directly applicable, due to the presence of phosphate buffer in the mobile phase. The effectiveness of a mobile phase composed by water and acetonitrile acidified with formic acid, in the ratio $H_2O/CH_3CN/HCOOH$ 64.9:35:0.1, was tested by the analysis of an orange fresh juice. In these conditions, by using a C18 column, synephrine eluted after 4.5 min (**Figure 4**), i.e., in a retention time about six times shorter than that observed in previously described conditions. Here, a classical C18 250 mm column was used, and a direct comparison with the chromatograms reported in **Figure 2**, usually adopted in quality control laboratories, can be done. It should be emphasized that the analysis time could be still reduced further 2−4-fold by using a 5−10 cm column.

The signals in the R_t range 15−22 min could be in principle due to material extracted from the cellulose acetate filter. In fact, it is known that this filter, treated with acetonitrile, can release many different compounds. However, the analysis of a sample obtained by filtering 10 mL of water:acetonitrile:formic acid 64.9:35:0.1 solution, led to a LC-MS chromatogram with very weak signals (10^4 counts) scattered in the R_t window 10−22 min. However, the possible coelution of synephrine with the components coming from the acetate filter as well as with other components of the natural extract cannot lead to any misleading result when MS^n is employed for synephrine quantification.

Once the analysis procedure to be followed was defined, a calibration curve was obtained by injection of known amounts of standard synephrine samples (in the range from 0.2 to 6.25

Scheme 1



μg/mL) and by measuring the peak areas related to the collisionally generated ions at *m/z* 150. The calibration curves showed good linearity over the full range tested. The linear regression parameters for the calibration curve, fitted by first-order polynomial model $y = a + bx$, were $a = 3.4$ and $b = 1.19$. The value of the correlation coefficient (R^2) was 0.9968.

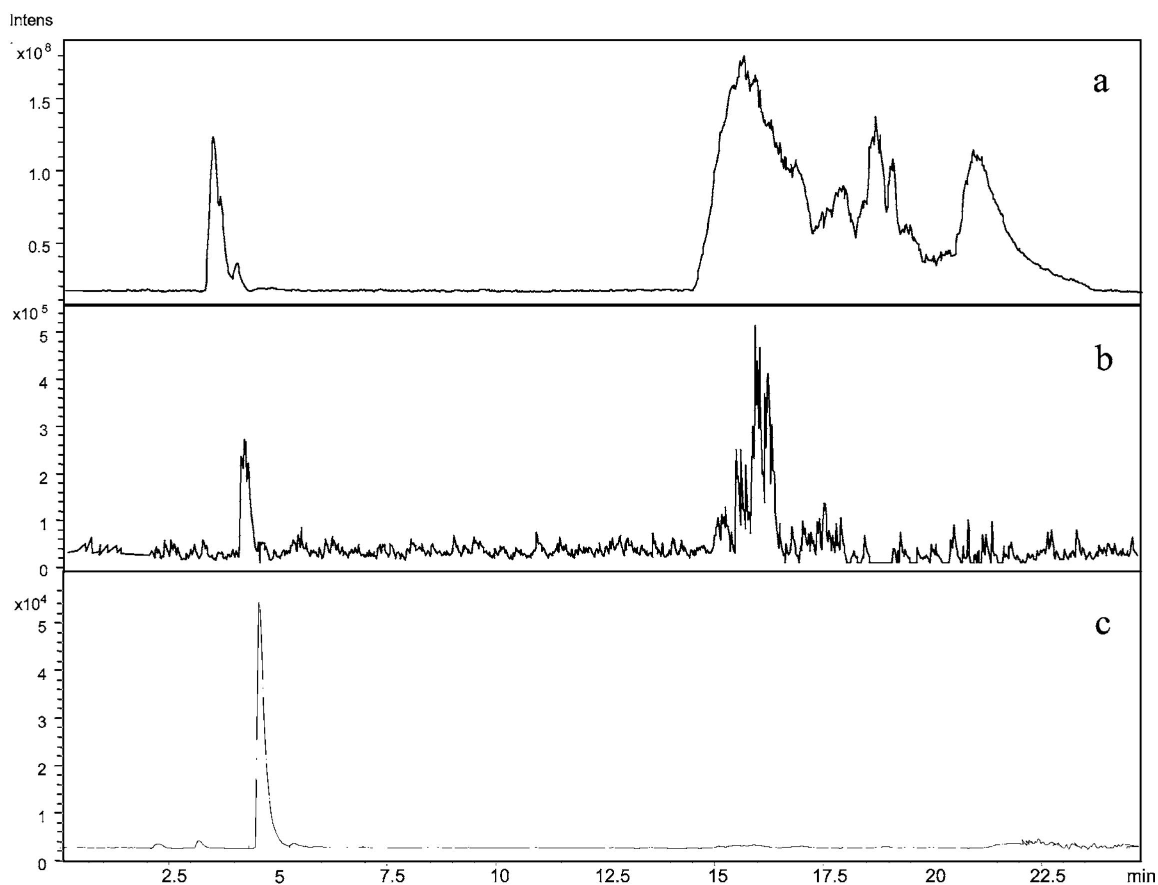


Figure 4. Total ion current chromatogram (**a**), extracted ion chromatogram of ion at *m*/*z* 168 (**b**) and of the ion at *m*/*z* 150 generated in a MS^2 experiment of orange [(*C. aurantium* L. var. *sinensis* (L.)] fresh juice (sample 1).

Table 2. *C. aurantium* L. Var. *Sinensis* (L.) (**a**) Oranges and (**b**) Orange Fresh Juices Synephrine Content[a]

Section a

sample	description	juice (μg/g)	pulp (μg/g)	juice + pulp (μg/g)
1	Tarocco fresh orange 1	26.65 ± 0.004	25.31 ± 0.11	26.15 ± 0.11
2	Tarocco fresh orange2	33.22 ± 0.15	37.81 ± 0.06	34.94 ± 0.16
3	Tarocco fresh orange 3	29.33 ± 0.12	30.06 ± 0.013	29.60 ± 0.12
4	Naveline fresh orange 1	13.80 ± 0.05	17.96 ± 0.3	15.4 ± 0.3
5	Naveline fresh orange 2	11.11 ± 0.14	15.61 ± 0.11	12.80 ± 0.18
6	Navel fresh orange 1	10.30 ± 0.013	18.02 ± 0.4	13.2 ± 0.4

Section b

sample	description	μg/g
7	commercial red orange fresh juice 1	29.9 ± 0.3
8	commercial red orange fresh juice 2	32.07 ± 0.19
9	commercial white orange fresh juice 1	12.97 ± 0.01
10	commercial white orange fresh juice 2	15.82 ± 0.05

[a] Data are given as means ± standard deviation of three independent batches.

The response linearity was tested also in the case of matrices (orange pulp and fresh orange juice) by adding known amounts of synephrine. In these cases, the linear regression parameters were as follows: $a = 4.1$ and $b = 0.98$ for orange pulp and $a = 4.2$ and $b = 0.95$ for juice matrix. The values of the correlation coefficients (R^2) were 0.9856 and 0.9806, respectively.

These good linear relationships suggested the possibility of a direct evaluation of synephrine without the use of any internal standard, just by spiking the samples of interest with known amounts of synephrine itself. The recovery capacities of this method, calculated by comparison of original samples and spiked samples, were 99.3%.

The so-developed LC/MS/MS method was applied to the analysis of the samples reported in **Table 2**, leading to total ion chromatograms analogous to that reported in **Figure 4a** (for example, juice of sample 1). By comparison with a standard synephrine sample, the peak for the drug would be present at $R_t = 4.5$ min, in the region in which at least three different components are detectable. In principle, reconstructed ion chromatograms related to ions of interest can be effective for its evaluation even in the presence of coeluting components. However, in this case, this approach is not particularly effective, as shown by the chromatogram reported in **Figure 4b**. Two isobaric species, with completely different retention times, are detectable. The R_t value of standard synephrine sample suggests that the peak at $R_t = 4.5$ min is that related to the analyte, but the quality of chromatographic data is inferred by the wide chemical background present in the chromatogram. Consequently, MS/MS experiments were considered essential for the unequivocal determination of synephrine peak as well as for quantitative purposes. By selection of the ion at m/z 168, its collision and the monitoring of the collisionally generated ion at m/z 150, the chromatogram reported in **Figure 4c** has been obtained, showing only one well-defined peak ($R_t = 4.5$ min) and a drastic reduction of the baseline noise. Looking at these results, the further analysis was performed following these last conditions.

***C. aurantium* L. Samples Analysis.** In the case of the immature bitter orange fruit dried extract (Advantra Z) standardized to 6% synephrine, the analysis performed in the LC-ESI/MS/MS conditions above-described leads us to determine a synephrine level of 6.27% (SD ± 0.035) while for the immature bitter orange fruit freeze-dried extract produced by Aboca, a synephrine content of 5.04% (SD ± 0.052) was determined.

Tarocco, naveline, and navel oranges, treated as described in the Materials and Methods, led to the values reported in **Table 2a**. In the same table (**Table 2b**), the levels determined for commercial fresh orange juices are also reported.

These data show that a higher content of synephrine was found in tarocco orange fresh-squeezed juice (26.65−33.22 μg/g) and in the corresponding separated pulp (25.31−37.81 μg/g) as well as in commercial red orange juices (29.9−32.07 μg/g) while lower synephrine levels were present in naveline and navel orange fresh-squeezed juices (10.30−13.80 μg/g), pulp (15.61−18.02 μg/g), as well in commercial white orange juices (12.97−15.82 μg/g) (**Table 2**).

To calculate the synephrine content in the original peeled oranges, we considered that juice and pulp were obtained with a yield of 62 ± 1 and 38 ± 1%, respectively. The same trend in the entire fruit (juice + pulp, **Table 2**) was observed.

The results obtained for all of the examined samples indicate that synephrine is present in both *C. aurantium* L. species (sweet and bitter) and it is distributed in all of the edible parts of the fruit; that is, synephrine is not only characteristic of the immature fruit of bitter orange but also of fresh oranges and orange juices, which are currently consumed in human feeding.

Conclusion. The LC-ESI/MS/MS method developed is rapid, simple, and allowed to obtain accurate evaluation of synephrine content in different type of *C. aurantium* L. samples without the need to separate this molecule from the other substances present in the complex matrix.

In principle, this method would permit an easy evaluation of synephrine in complex mixtures such as herbal preparations, where *C. aurantium* L. sample is mixed with other herbs, which potentially might interfere with the correct evaluation of synephrine.

This analytical method has a particular relevance in consideration of presumed health risks associated with synephrine consumption, as evidenced by the Italian Ministry of Health (Directive of July 18, 2002) with the limit of 30 mg daily dose and the warning: "In presence of cardiovasculopathies and/or arterial hypertension, before consuming the product, consult the doctor". In this frame, herbal product manufacturers would employ effective analytical methods for the evaluation of synephrine levels in the commercial final products, so to make consumers conscious of the products that they are assuming.

The present investigation put in evidence an intriguing point related to synephrine intake in a normal diet, i.e., by direct assumption of fresh oranges and commercially available orange

juices. Indeed, for example, considering the synephrine total content (from juice + pulp) of tarocco oranges (i.e., sample 2), corresponding to 0.00349% in weight, and considering that the weight of two oranges without peel is approximately 350 g, it follows that by eating two oranges in a day, 12.21 mg of synephrine is ingested. Obviously, there are notable differences in the synephrine concentration between unripe bitter orange fruits that contain 0.1% synephrine (*6*) and sweet orange edible fruit that contain 0.0013−0.0035% (13−35 ppm) synephrine.

The method here described allows a rapid and unequivocal determination of synephrine levels directly in orange fresh juices and pulp, in commercially available orange juices, and in herbal preparations from *C. aurantium* L. Works are now in progress based on the use of this method in complex herbal preparations in which *C. aurantium* L. is only a minor component.

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LITERATURE CITED

(1) Swingle, W. T.; Reece, P. C. In *The Botany of Citrus and Its Wild Relatives*; Reuther, W., Webber, H., Bachelor, L., Eds.; The Citrus Industry: University of California, 1967; Vol. 1, pp 190−430.

(2) Cobley, L. S. *An Introduction to the Botany of Tropical Crops*; Longmans: London, 1956; p 266.

(3) Penso, G. *Index Plantarum Medicinalium Totius Mundi Eorumque Synonymorum*, 2nd ed.; O. E. M. F. Int.: Milano, 1997; pp 286−288.

(4) Trease, G. E.; Evans, W. C. Bitter orange peel. In *Trease and Evans Pharmacognosy*, 15th ed.; W. B. Saunders: London, Philadelphia, Baillière Tindall, 1989; pp 266−267.

(5) Leung, A. Y.; Foster, S. Orange (bitter and sweet). In *Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics*, 2nd ed.; John Wiley & Sons Inc.: New York, 1996; pp 393−397.

(6) Immature orange fruit. In *Herbal Drugs and Phytopharmaceuticals, a Handbook for Practice on a Scientific Basis*, 3rd ed.; Wichtl, M., Ed.; Medpharm: Stuttgart; CRC Press: Boca Raton, London, 2004; p 71.

(7) Orange peel, bitter. In *Herbal Medicine*−*Expanded Commission E Monographs*, 1st ed.; Blumenthal, M., Goldberg, A., Brinckmann, J.. Eds.; American Botanical Council, Austin, TX; Integrative Medicine Communications Publisher: Newton, MA, 2000; pp 287−289.

(8) Wheaton, T. A.; Stewart, I. Quantitative analysis of phenolic amines using ion-exchange chromatography. *Anal. Biochem.* **1965**, *12*, 585−592.

(9) Wheaton, T. A.; Stewart, I. The distribution of tyramine, *N*-methyltyramine, hordenine, octopamine, and synephrine in higher plants. *Lloydia* **1970**, *3*, 244−254.

(10) Preuss, H. G.; DiFernando, D.; Bagchi, M.; Bagchi, D. *Citrus aurantium* as a thermogenic, weight-reduction replacement for Ephedra: An overview. *J. Med.* **2002**, *33*, 247−264.

(11) Fough-Berman, A.; Myers, A. *Citrus aurantium*, an ingredient of dietary supplements marketed for weight loss: Current status of clinical and basic research. *Exp. Biol. Med*. **2004**, *229*, 698−704.

(12) Calapai, G.; Firenzuoli, F.; Saitta, A.; Squadrito, F.; Arlotta, M. R.; Costantino, G.; Inferrera, G. Antiobesity and cardiovascular toxic effects of *Citrus aurantium* extracts in the rat: A preliminary report. *Fitoterapia* **1999**, *70*, 586−592.

(13) Huang, Y. T.; Wang, G. F.; Chen, C. F.; Chen, C. C.; Hong, C. Y.; Yang, M. C. Fructus aurantii reduced portal pressure in portal hypertensive rats. *Life Sci*. **1995**, *57*, 2011−2020.

(14) Hofstetter, R.; Kreuder, J.; Von Bernuth, G. The effect of oxedrine on the left ventricle and peripheral vascular resistance. *Arzneimittelforschung* **1985**, *35*, 1844−1846.

(15) AHPA News 4, September 2, 2004 [online http://www.ahpa.org/members/update_04_0902.htm].

(16) Penzak, S. R.; Jann, M. W.; Gold, J. A.; Hon, Y. Y.; Desai, H. D.; Gurley, B. J. Seville (sour) orange juice: Synephrine content and cardiovascular effects in normotensive adults. *J. Clin. Pharmacol.* **2001**, *41*, 1059−1063.

(17) Colker, C. M.; Kalman, D. S.; Torina, G. C.; Perlis, T.; Stress, C. Effects of *Citrus aurantium* extract, caffeine, and St. John's Wort on body fat loss, lipid levels and mood states in overweight healthy adults. *Curr. Ther. Res.* **1999**, *60*, 145−152.

(18) Kusu, F.; Matsumoto, K.; Azai, K.; Takamura, K. Determination of synephrine enantiomers in food and conjugated synephrine in urine by high-performance liquid chromatography with electrochemical detection. *Anal. Biochem.* **1996**, *235*, 191−194.

(19) Niemann, R. A.; Gay, M. L. Determination of ephedrine alkaloids and synephrine in dietary supplements by column-switching cation exchange high-performance liquid chromatography with scanning-wavelength ultraviolet and fluorescence detection. *J. Agric. Food Chem.* **2003**, *51*, 5630−5638.

(20) Takei, H.; Hirabuki, M.; Yoshizaki, F. Analysis of synephrine in the peel of citrus fruit, immature citrus fruit and decoctions of Chinese medicinal prescriptions containing these crude drugs by capillary electrophoresis. *Anal. Sci.* **1999**, *15*, 1017−1020.

(21) Schaneberg, B. T.; Khan, I. A. Quantitative and qualitative HPLC analysis of thermogenic weight loss products. *Pharmazie* **2004**, *59*, 819−823.

(22) Pellati, F.; Benvenuti, S.; Melagari, M.; Firenzuoli, F. Determination of adrenergic agonists from extracts and herbal products of *Citrus aurantium* L. *var. amara* by LC. *J. Pharm. Biomed. Anal.* **2002**, *29*, 1113−1119.

(23) Pellati, F.; Benvenuti, S.; Melegari, M. High-performance liquid chromatography methods for the analysis of adrenergic amines and flavanones in *Citrus aurantium L.* var. *amara*. *Phytochem. Anal.* **2004**, *15*, 220−225.

(24) Gay, M. L.; Niemann, R. A.; Musser, S. M. *An LC/MS/MS Method for the Determination of Synephrine and Ephedrine-Type Alkaloids in Dietary Supplements Using a Stable-Isotope Labeled Internal Standard*, 9th Annual FDA Science Forum "FDA Science: Protecting America's Health" April 24−25, 2003, Category A: Methods Development and Application: Analytical Chemistry, Board A-22.

(25) Karni, M.; Mandelbaum, A. *Org. Mass Spectrom*. **1980**, *15*, 53−64.

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