Food Additives & Contaminants: Part B: Surveillance

Aminocarminic acid in E120-labelled food additives and beverages

Leonardo Sabatino a, Monica Scordino a, Maria Gargano a, Francesco Lazzaro a, Marco A. Borzì a, Pasqualino Traulo a & Giacomo Gagliano a

a Ministero delle Politiche Agricole Alimentari e Forestali (MIPAAF), Dipartimento dell’Ispettorato centrale della tutela della qualità e della repressione frodi dei prodotti agroalimentari (ICQRF), Laboratory of Catania, Catania, Italy

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Aminocarminic acid in E120-labelled food additives and beverages

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Ministero delle Politiche Agricole Alimentari e Forestali (MIPAAF), Dipartimento dell’Ispettorato centrale della tutela della qualità e della repressione frodi dei prodotti agroalimentari (ICQRF), Laboratory of Catania, Catania, Italy

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An analytical method was developed for investigating aminocarminic acid occurrence in E120-labelled red-coloured beverages and in E120 additives, with the aim of controlling the purity of the carmine additive in countries where the use of aminocarminic acid is forbidden. The carminic acid and the aminocarminic acid were separated by high-performance liquid chromatography–photodiode array–tandem mass spectrometry (HPLC-PDA-MS/MS). The method was statistically validated. The regression lines, ranging from 10 to 100 mg/L, showed \( r^2 > 0.9996 \). Recoveries from 97% to 101% were obtained for the fortification level of 50 mg/L; the relative standard deviations did not exceed 3%. The LODs were below 2 mg/L, whereas the LOQs did not exceed 4 mg/L. The method was successfully applied to 27 samples of commercial E120-labelled red-coloured beverages and E120 additives, collected in Italy during quality control investigations conducted by the Ministry. The results demonstrated that more than 50% of the samples contained aminocarminic acid, evidencing the alarming illicit use of this semi-synthetic carmine acid derivative.

**Keywords:** acid-stable carmine; 4-aminocarminic acid; carminic acid; carmine; cochineals; mass spectrometry; pigments; food colourants

**Introduction**

Among additives, colourants are added to food for one or more of the following reasons: to replace colour loss during processing, to enhance colour already present, to minimise batch-to-batch variations and to colour otherwise uncoloured food (Ash and Ash 1995). Food colours can be divided into four categories: natural, nature-identical, synthetic and inorganic colours (Scotter 2011). Natural colours are pigments made by living organisms. Among them, carminic acid (E120) is a red glucosidal hydroxyanthrapurin that occurs naturally in some insect scales, such as *Dactylopius coccus* and *Polish cochineal*. The chemical structure of carminic acid consists of an anthraquinone core, linked to a glucose sugar unit (Dapson 2007; Scotter 2011). Carmines and carminic acid are obtained from aqueous, aqueous alcoholic or alcoholic extracts from cochineal, which consists of dried bodies of the female insect *D. coccus* Costa. In commercial products, the colouring principle is present in association with ammonium, calcium, potassium or sodium cations, single or in combination, and these cations may also be present in excess. Commercial preparations of carmine may contain not less than 2.0% carminic acid in extracts containing carminic acid and not less than 50% carminic acid in chelates (European Commission 2012). Cochineal, for which a provisional ADI of 2.5 mg/kg body weight has been established, is important for the food industry in the production of alcoholic and flavoured drinks. The use of E120 has increased in recent years since EU Regulation 1333/2008, requiring the mandatory statement “may have an adverse effect on activity and attention in children” on products that contain E102, E104, E110, E122, E124 and E129 colorants, came into force (European Commission 2008). Recovery and purification of carminic acid from raw cochineal is regarded as a difficult and complicated process. Current industrial technology suffers from low and irreproducible yields, while generating a low-quality final product (Borges et al. 2012).

The International Numbering System for approved food colours in the countries of the European Union and elsewhere lists E120 for carminic acid, specifying the molecular formula (C_{22}H_{20}O_{13}) and molecular weight (492.39) of the molecule responsible for the colour (Table 1). Dapson (2005, 2007) noticed that many E120 additives contained substantial amounts of aminocarminic acid, a synthetic derivative obtained after heating carminic acid with ammonia. Different from carminic acid, aminocarminic acid maintains its deep red colorant power at very low pH and it is sold...
Table 1. Physical-chemical characteristics for carminic and 4-aminocarminic acid.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Carminic acid</th>
<th>4-aminocarminic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical name</td>
<td>7-α-D-Glucopyranosyl-9, 10-dihydro-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-2-anthracencarboxylic acid</td>
<td>7-α-D-Glucopyranosyl-9, 10-dihydro-5-amino-3,6,8-trihydroxy-1-methyl-9,10-dioxo-2-anthracencarboxylic acid</td>
</tr>
<tr>
<td>Chemical structure</td>
<td><img src="image1.png" alt="Carminic Acid Structure" /></td>
<td><img src="image2.png" alt="4-Aminocarminic Acid Structure" /></td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₂₂H₂₀O₁₃</td>
<td>C₂₂H₂₁NO₁₂</td>
</tr>
<tr>
<td>Retention time</td>
<td>30.6 min</td>
<td>36.9 min</td>
</tr>
<tr>
<td>UV₅₆₅ (pH 1)</td>
<td>495 nm</td>
<td>530 nm, 565 nm</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>492.4 u</td>
<td>491.4 u</td>
</tr>
<tr>
<td>ESI-MS/MS fragmentation</td>
<td>491 &gt; 447 loss of CO₂</td>
<td>490 &gt; 472 loss of H₂O and/or NH₃, 490 &gt; 400 loss of CO₂ and H₂O or NH₃ and CO or C₂H₂, 490 &gt; 446 loss of CO₂</td>
</tr>
<tr>
<td>Elemental composition</td>
<td>C = 54%, H = 4%, O = 42%</td>
<td>C = 54%, H = 4%, O = 39%, N = 3%</td>
</tr>
</tbody>
</table>

as “acid-stable carmine” suitable for acidic food, even if it is not approved for use in food. The aminocarminic method of preparation was illustrated in a United States Patent (Schul 1992) and molecular formula (C₂₂H₂₁NO₁₂, molecular weight 491.10) was firstly elucidated by Sugimoto et al. (2002).

These few scientific references have elucidated spectroscopic and chemical characteristics of aminocarminic acid, but no method has been developed to detect its presence in products in which this compound could be found. With the aim of gaining a greater understanding on the conformity and legal compliance of E120 additives used in food preparations, in this study a suitable method for the detection of carminic acid and aminocarminic acid in samples of red-coloured beverages containing E120 as ingredient and in E120 additives has been developed. The method was validated in terms of specificity, linearity, precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ). The proposed analytical protocol is currently applied in ICQRF Catania laboratory, in the frame of an Italian Ministry quality control additives investigation.

Materials and methods

Chemicals

Carminic acid (purity > 99%) was purchased from Chem Service (Milan, Italy). 4-Aminocarminic acid was synthesised as reported by Sugimoto et al. (2002), crystallised step by step with 2N HCl, water excess eliminated by lyophilisation and its identity confirmed by direct infusion into the LC/MS² equipment and by CHNS elemental analysis. Relative purity (>98%) was determined by high-performance liquid chromatography–photodiode array–tandem mass spectrography (HPLC-PDA-MS/MS).

HPLC-grade acetonitrile and formic acid were supplied by Romil (Milan, Italy); citric acid, hydrochloric acid and ammonium hydroxide were purchased from Carlo Erba (Bologna, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA (Millipore; Vimodrone, MI, Italy) purification system.

Standards

Working standard solutions were prepared each time by dissolving about 0.001 g of standard additives in 10 mL of methanol. Stock solutions stored at −18°C were stable at least 6 months.

Sampling

A total of 15 samples of E120 additives and 12 samples of commercial red-coloured beverages containing E120 as ingredients were collected in Italy in the framework of a 12-month quality control investigation performed by the Italian Ministry of Agricultural Alimentary and Forestry Policies during the years 2011 and 2012. As per the regulations, five subsamples were taken, labelled with a description of location, date and typology and an official report was written.
Four subsamples were sent to Catania Laboratory of Central Inspectorate Department of Protection and Prevention of Fraud, Quality of Food Products (ICQRF) for analysis, where a certificate of analysis was released. Analysis was carried out in duplicate. In case of additives, about 50 mg of sample was weighed and dissolved in 1000 mL water. In case of beverages, no pre-treatment of the sample was necessary. The solutions were filtered with 0.45-μm PTFE filter (VWR, Milan, Italy) and directly analysed. Quantitative results were expressed in terms of mean value (% weight/weight for additives and mg/L for beverages), and relative uncertainty was estimated by the Horwitz approach (Harris 2006).

**HPLC/PDA/MS-MS**

The analyses were performed with a liquid chromatograph equipped with an Aquasil C18 150 × 2.1 mm i.d. 3-μm analytical column, a Finnigan Surveyor MS pump, autosampler, PDA, and LCQ DECA XP MAX ion trap detector, operated with TunePlus Excalibur software (Thermo Fisher Scientific, Waltham, MA, USA). Eluent flow rate was 200 μL/min, column temperature 30°C and the injection volume 10 μL. A binary gradient of 0.3% formic acid in water (A) and 0.3% formic acid in acetonitrile (B) was used. The mobile-phase gradient program was as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60–65 min, 43% B; 70–80 min, 5% B. The wavelength range examined by the photodiode-array detector was 200–750 nm. Mass spectral analyses were performed operating in negative electrospray ionisation (ESI) mode using the following MS settings: needle voltage 3.5 kV, capillary voltage 18 V, the capillary was heated at 250°C, sheath gas flow rate 36 and auxiliary gas 14 arbitrary units. MS-MS spectra were obtained using collision energy at 30% of instrument maximum, revealing precursor ions for aminocarminic acid at 490 and 525 nm, respectively. Mass scans in negative ESI mode were performed with a soft electrospray, showing optimal fragmentation patterns at a collation energy level of 30% of the instrument maximum, revealing precursor ions for aminocarminic acid at m/z 490 u and of 491 u for carminic acid, respectively. The fragmentation pattern showed following differences for the two molecules: carminic acid only lost CO2 (m/z 447), while amino-carminic acid showed the loss of CO2 (m/z 446), the simultaneous loss of H2O and/or NH3 (m/z 472) and an additional fragment at m/z 400 (loss of CO2 and H2O or NH3 and CO or C2H2). The characteristics of carminic acid were in good agreement with those reported by Feng et al. (2011), who analysed 40 dyes in soft drinks by LC-ESI-MS, but without paying attention to aminocarminic acid. Under the described chromatographic conditions, the carminic and aminocarminic acid were well separated and easily identified by UV–Vis spectra and the fragmentation pattern of the parent ions (Figure 1).

**Results and discussion**

**Method development**

In order to develop a sensitive method to identify the major fragments after collisions, at first mass characterisation was studied by direct injection of stock solutions of carminic and aminocarminic acid. Mass scans in negative ESI mode were performed with a soft electrospray, showing optimal fragmentation patterns at a collation energy level of 30% of the instrument maximum, revealing precursor ions for aminocarminic acid at m/z 490 u and of 491 u for carminic acid, respectively. The fragmentation pattern showed following differences for the two molecules: carminic acid only lost CO2 (m/z 447), while amino-carminic acid showed the loss of CO2 (m/z 446), the simultaneous loss of H2O and/or NH3 (m/z 472) and an additional fragment at m/z 400 (loss of CO2 and H2O or NH3 and CO or C2H2). The characteristics of carminic acid were in good agreement with those reported by Feng et al. (2011), who analysed 40 dyes in soft drinks by LC-ESI-MS, but without paying attention to aminocarminic acid. Under the described chromatographic conditions, the carminic and aminocarminic acid were well separated and easily identified by UV–Vis spectra and the fragmentation pattern of the parent ions (Figure 1). Spectroscopic characteristics of both analytes were in agreement with literature data (Schul 1992; Sugimoto...
et al. 2002; Dapson 2007). Under acidic conditions, carminic acid had a broad absorbance peak at 490 nm, while the aminocarminic acid had a main absorbance peak at 525 nm with a secondary peak about 35 nm further in the spectrum. These UV absorbances are in agreement with Samari et al. (2010), who published the influence of pH on UV absorbance of carminic acid.

Figure 1. HPLC-PDA chromatogram, UV-Vis spectra and ESI/MS-MS fragmentation patterns of carminic acid (A, A') and 4-aminocarminic acid (B, B').
Method validation

Analytical parameters of the proposed method were evaluated according to the criteria given above. Results are reported in Table 2. Specificity of the method was good. No interferences due to beverage or additive matrices were found. Hence, no further concentration/cleanup pretreatment was required. LOQs obtained in the proposed method are compatible with E120 concentration levels generally used in beverages (e.g. a legal limit of 100 mg/L). The precision of the method was good, not exceeding a coefficient of variation of 3%. The AOAC’s Single Lab Validation document recommends general recovery limits of 80%–110% with precision of about 6% at the investigated concentration levels (AOAC 2002). Thus, from the data in Table 2, it can be concluded that the method could be considered sufficiently accurate and precise for the purpose.

Occurrence of aminocarminic acid in E120-labelled food additives and beverages

The developed analytical method has been applied for the routine analysis of 27 samples in the ICQRF Laboratory of Catania to investigate the occurrence of aminocarminic acid in red-coloured beverages containing E120 (12 samples) as ingredient and in E120 additives as such (15 samples). Samples were processed as previously described and the results are reported in Table 3. Analysis of the 12 red-coloured beverages showed the presence of aminocarminic acid in 6 samples, which is almost always associated with
minimum quantities of carminic acid, probably as un-reacted residue. Although aminocarminic acid is a non-permitted colour additive in the food industry, the found amount never exceeded the legal limits set for permitted colorants in beverages. As regards the analysis of the colour additives, a rate of non-compliance of 60% was determined. Nine of the tested additives were composed almost exclusively of aminocarminic acid, whereas residual quantities of un-reacted carminic acid have been found in almost all samples. All additives not conforming to the EU regulation on additives were sold as “acid-stable carmine,” although the leaflets reported the percentage of carminic acid as the molecule responsible for the colour. These results proved the excellent performance of the analytical protocol on matrices beyond lab-prepared and fortified samples and revealed alarming illegal presence of aminocarminic acid in E120-labelled colorants.

Conclusions
In this study, a validated chromatographic method was developed to be used for the identification of aminocarminic acid in food colours labelled as E120. The absence of a clean-up step turns in recovery’s advantage and permits cheaper analysis, low environmental impact and faster results, making the method favourable for routine application. Sensitivity, precision and accuracy of the method were adequate for the scope. Application of the method to samples of Italian beverages and dyes showed the widespread presence of this semi-synthetic carmine acid derivative, which is not permitted by law in food industry, with unknown effects on human health. Furthermore, since colorants came from international suppliers, the problem may be present in other markets and in other food products (e.g. candies, cakes, ice creams). Results of this study give support to the hypothesis of Dapson (2007) for possible improper use of aminocarminic acid as carminic acid, which needs consideration by risk assessors and organisations with an interest in protecting consumer’s health.

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References