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Liquid chromatography with accurate mass measurement on a triple quadrupole mass-spectrometer for the identification and quantification of *N*-lactoyl ethanolamine in wine

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In this study a specific taste-modulating flavor ingredient, *N*-lactoyl ethanolamine, was determined in two Beerenauslese wines using preparative LC, as a first isolation and concentration step, followed by LC-MS/MS on a triple quadrupole in accurate mass (AM) mode. The accurate masses of the analyte and three characteristic fragments were determined with mass accuracies between 8 and 20 ppm. *N*-lactoyl ethanolamine concentrations in the wines were 0.4 and 2.5 mg/L.

Keywords: Accurate mass / Liquid chromatography / Mass spectrometry / *N*-lactoyl ethanolamine / Wine

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1 Introduction

In the last decades research in flavor chemistry was mainly focused on the elucidation of the composition of the volatile fraction of fruits or foodstuff because this part causes the smell. Especially the development of high-resolution GC coupled with MS (HRGC-MS) towards greater sensitivity formed the basis for discovery of more and more potent flavor compounds at trace level [1, 2]. It is known that, apart from the volatile flavor fraction, the perception of foodstuff in the mouth is an important factor for the sensation of a harmonic and "complete" food product. Five characteristics for taste are commonly described – sweet, sour, bitter, salt, and umami – caused by, *e.g.*, sugar, acids, caffeine, sodium chloride, or mono sodium-glutamate [3].

The latest research on human receptor physiology has shown that there is a number of different receptor types present in the mouth and on the tongue, which are involved in the perception of taste [4]. The actual challenge for the flavor industry today is to discover molecules in foodstuff, which are triggering these receptors. This will provide the opportunity to create a new generation of flavors, which have an additional taste effect. Those flavors can contribute to actual nutritional problems by creating low-salt, low-fat, or low-sugar food products without compromising on taste. Compared with classical volatile flavor compounds, taste molecules are usually relatively polar and have a higher molecular weight, which requires LC for their chromatographic separation. Since years LC coupled with MS (LC-MS) is a standard tool for drug discovery and metabolic studies in the pharmaceutical industry. Especially the possibility to measure the accurate mass (AM) of target molecules provides additional and essential information about the molecular structure [5, 6].

So far, AM measurements for determining elemental compositions, elucidating the structure of unknown compounds and identifying metabolites and impurities, were mainly performed on TOF instruments [7, 8]. Recently, a new generation of triple-quadrupole mass spectrometers was developed. These instruments combine the strength of triple-quadrupole systems – to perform MS/MS fragmentation experiments and the possibility of reliable quantification of target compounds – with the new and exciting possibility to measure the AM within the third quadrupole. AM measurements using a triple-quadrupole instrument have been applied for determining elemental compositions, elucidating the structure of unknown compounds, and identifying metabolites and impurities [9, 10].

As a result of our screening project for taste-modulating substances, *N*-lactoyl ethanolamine was identified to have tingling character and delivers a refreshing effect. Based on a literature survey white wine was selected as a possible

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Abbreviations: AM, accurate mass; **ELSD,** evaporative light scattering detector; **PEG,** polyethyleneglycol; **SRM**, selected reaction-monitoring

source for *N*-lactoyl ethanolamine because it contains ethanolamine and ethyllactate, potential precursors [11, 12]. The experiments detailed in this work demonstrate that the combination of preparative LC – as a first isolation and concentration step – followed by LC–AM MS is a very efficient approach to study a polar taste active molecule in foodstuff, even at trace level.

2 Materials and methods

2.1 Chemicals

Prepsolv and lichrosolv ACN, lichrosolv water, and methanol and formic acid were purchased from Merck (Darmstadt, Germany), ammonium formate from Sigma–Aldrich (Steinheim, Germany). Ethanolamine was obtained from Quaron (Zwijndrecht, The Netherlands) and ethyl lactate from Purac Biochem BV (Gorinchem, The Netherlands). Water for preparative HPLC (0.5 MΩ) was prepared with a Millipore-Elix system (Etten-Leur, the Netherlands). The wine fractions were prepared from two Beerenauslese wines, a 2003 Weinlaubenhof Kracher Burgenland Beerenauslese Cuvée and a 2001 Ilbesheimer Herrlich Beerenauslese PFALZ that were bought in a local winestore in Laren, the Netherlands.

2.2 Preparation of 2-hydroxy-*N*-(2-hydroxyethyl)-propanamide (*N*-Lactoyl ethanol amine)

Fifty grams of ethanolamine and 150 g of ethyl lactate were mixed together in a flask connected to a distillation unit. The reaction mixture was heated to 110°C while stirring. During the reaction ethanol was formed which started to distill off at 105°C. After 1.5 h no more ethanol was obtained and the reaction mixture was cooled to 85°C. After cooling, the pressure was reduced stepwise to 6 mbar while heating the reaction vessel to 150°C to distill off the excess ethyl lactate. The obtained reaction product was analyzed with NMR.

^{1}H and ^{13}C NMR spectra were recorded with an ECA 600 MHz NMR spectrometer (JEOL, Tokyo, Japan) equipped with a 5 mm autotune probe. D_2O was used as solvent; resonance frequencies are relative to the internal standard d4trimethylsilyl propionate, sodium salt (TSP). The following proton and carbon NMR signals were obtained: ^{1}H: 1.38 ppm (d, 3H, J = 7.15 Hz), 3.39 ppm (t, 2H, J = 5.50) 3.68 ppm (t, 2H, J = 5.50), 4.27 ppm (q, 1H, J = 6.87 Hz), ^{13}C: 22.44 ppm (C-9); 43.83 ppm (C-3); 62.74 ppm (C-2); 70.52 ppm (C-7); 180.58 ppm (C-5). The obtained product was identified as 2-hydroxy-*N*-(2-hydroxyethyl)propanamide (*N*-lactoyl ethanolamine) and was found to be ~97% pure.

2.3 Preparative LC fractionation

For preparative LC a Gilson (Villiers le Bel, France) pump model 321 with a liquid handler model 215 were used equipped with an evaporative light scattering detector (ELSD) model 2000ES (Alltech, Deerfield, IL, USA) detector set at 81.3°C and the helium flow at 2.1 L/min. The separation was carried out on a 300 mm × 20 mm ID 9 µm Prevail carbohydrate ES column (Altech, Deerfield, IL, USA). Isocratic elution was carried out with water:ACN 20:80 (% v/v), at a flow rate 15 mL/min. Of the untreated wine 200 µL was injected onto the system and fractions were collected in the 6–7 min range. Each wine was injected 50 times and combined fractions were evaporated to 0.5 mL under reduced pressure at 40°C. The obtained extracts were filtered over a 0.45 µm filter and 10 µL was injected onto the LC-MS system.

2.4 LC–ESI-MS

For LC-MS a ThermoFinnigan (San Jose, CA, USA) Surveyor LC system was used equipped with an MS pump and a PDA/UV detector. The set-up was coupled to a ThermoFinnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer. The separation was carried out on a 150 mm × 2.1 mm ID 5 µm Atlantis C18 column (Waters, Milford, MA, USA). The LC eluent consisted of (A) 10 mM aqueous ammonium formate buffer and (B) 10 mM aqueous ammonium formate buffer in 80% ACN (both pH 4). Gradient elution of 100% A to 100% B in 10 min, followed by an isocratic step of 100% B over 5 min, was performed at a flow rate of 0.2 mL/min. ESI spectra were obtained in the positive ionization mode in the *m/z* 50–1000 range. The capillary temperature was set at 270°C and the capillary voltage at 4000 V; the sheath- and auxillary-gas (nitrogen) flow rates were 80 and 40 U, respectively, which corresponds to approximately 200 and 20 L/h.

For the AM experiments, ammoniated polyethyleneglycol (PEG) was used as an internal lockmass solution. A 50 nM PEG in methanol:water (1:1 v/v) solution was infused post-column into the LC flow at a flow rate of 20 µL/min.

3 Results

3.1 Preparative LC fractionation

To determine the retention time of *N*-lactoyl ethanolamine, the wine was spiked with a 10 ppm standard solution and injected in triplicate (results not shown). Consequently, the untreated wines were injected and fractions were collected between 6 and 7 min. The LC–ELSD chromatogram of the Ilbesheimer wine is shown in Fig. 1. As it can be seen in the

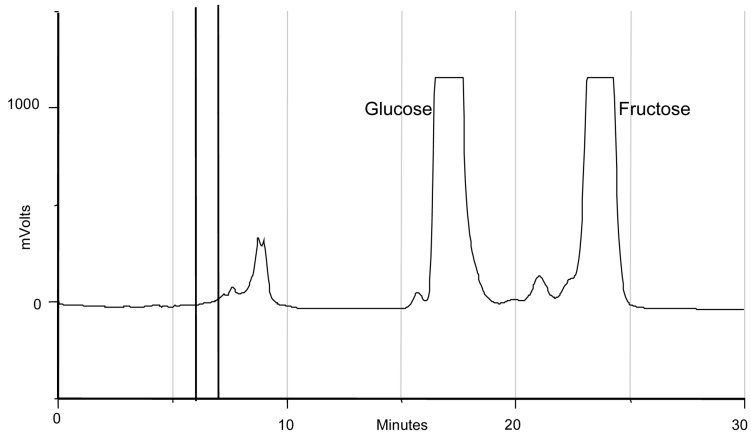

Figure 1. LC–ELSD of Ilbesheimer wine. For LC conditions, see text.

figure, in the 6–7 min region no pronounced peaks were observed. Therefore, the fraction collection was repeated 20 times, which, after evaporation of the solvent, resulted in 20-fold higher concentration for the Ilbersheimer wine. The Burgenland wine was treated similarly achieving a 10-fold concentration.

The large peaks between 16–18 and 23–25 min are contributed to glucose and fructose, respectively. Their concentrations were 2 and 7% in the Burgenland and 3 and 6% in the Ilbesheimer wine, respectively.

3.2 AM MS

To perform AM measurements on a triple quadrupole instrument several calibration steps are needed. Firstly, the Digital-Analog-Converter (DAC) was once calibrated in the factory for 20+ bits of linearity at stable temperature; secondly, the mass axis was coarsely calibrated in the laboratory at only two points using standard tuning and calibration procedures; and, thirdly, a "super calibration" was performed in which the mass axis was linearized with ammoniated PEG to compensate for all system nonlinearities. Furthermore, during the measurements, a lock mass solution was continuously introduced into the MS, together with the LC-flow, to refine the final AM measurement. In the current study, ammoniated PEG clusters and their fragments – with known *m/z* values – were used as lock ions. The deviation between their measured and known *m/z* values was used to continuously correct the measurement of the (fragment) ions of the analyte.

To obtain good MS/MS spectra of both the analyte and the ammoniated PEG clusters, the collision energy was optimized between 10 and 50 eV by direct infusion of 10 mg/L aqueous solutions of *N*-lactoyl ethanolamine together with 50 nM ammoniated PEG, each at 20 µL/min. The source temperature and spray voltage were optimized to 275°C and 5000 V, respectively. For both ammoniated PEG and the

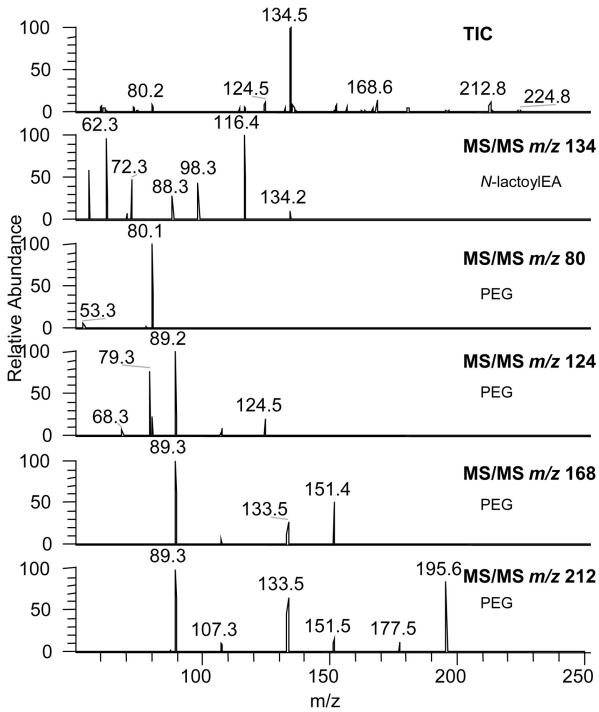

Figure 2. ESI(+)-MS/MS of *N*-lactoyl ethanolamine and ammoniated PEG. For further details, see text.

analyte, the highest signal in selected reaction-monitoring (SRM) acquisition was obtained at 15 eV. The obtained MS/MS spectra of *N*-lactoyl ethanolamine and of four ammoniated PEG clusters are shown in Fig. 2. The characteristic fragments *m/z* 88, 98, and 116 (for accurate masses and elemental compositions, see Fig. 3) in the MS/MS spectrum of *N*-lactoyl ethanolamine (second trace of Fig. 2) were selected for monitoring in the isolated wine fractions. Figure 4 shows the structures and accurate masses of the ammoniated PEG clusters and their fragments; in bold are the fragments that were used as lockmasses in the MS/MS experiments of the wine fractions, described below.

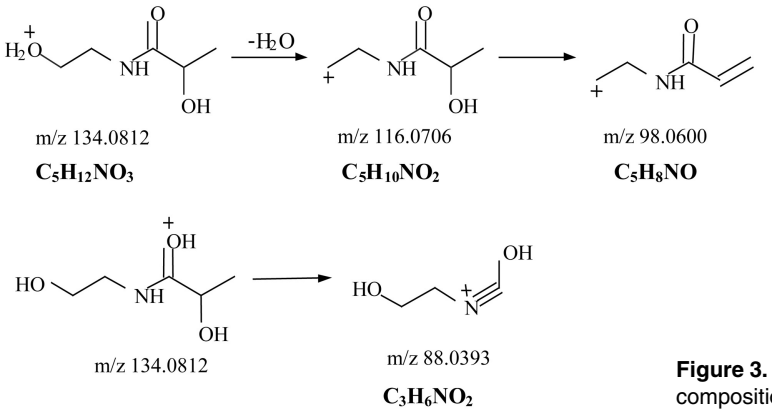

Figure 3. *N*-lactoyl ethanolamine fragment ions with elemental composition and accurate masses.

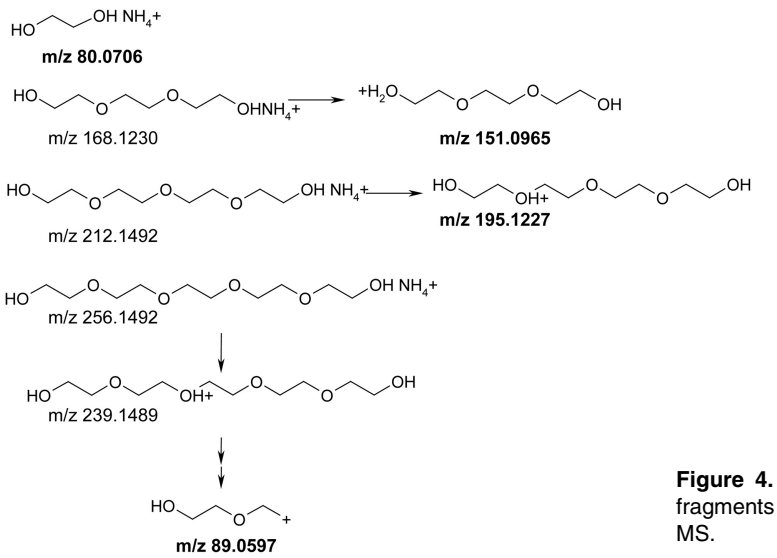

Figure 4. Ammoniated PEG fragment ions. Masses of the fragments indicated in bold were used as lock masses in MS/MS.

AM measurements of the isolated fractions were performed in SRM mode. In SRM, MS/MS transitions of both the analyte and the lockmasses were monitored and the *m/z* values of the analyte fragments were continuously corrected with the data of the ammoniated PEG fragments as described previously. The LC–SRM-MS/MS chromatogram of the Burgenland wine fraction is shown in Fig. 5A. The spectrum of the peak at 6.8 min is shown in Fig. 5B. For each fragment ion, the measured AM, the three closest elemental compositions, and their mass accuracy are shown. The structures, masses, and accuracy (between 8 and 20 ppm in all cases) of the proposed *N*-lactoyl ethanolamine fragments are indicated in the figure. Based on these results and the AM spectrum of the reference standard, the peak at 6.7 min was identified as *N*-lactoyl ethanolamine. The relative abundance of the fragments of the analyte *m/z* 98 and *m/z* 88 to the most intensive fragment *m/z* 116 was 7 and 3%, respectively. This was in very good agreement with the fragmentation pattern of the reference standard (8 and 3%). In the same way, *N*-lactoyl ethanolamine was identified in the Ilbesheimer wine (results not shown).

The linearity of MS detection was tested in SIM mode at unit mass resolution (The first (Q1) and the third quadrupole (Q3) set at 0.7 full width half mass height (FWHM)) and in SRM in AM mode (Q1 set at 0.7 FWHM, Q3 at 0.1). R^2 values and LODs (S/N = 3) were determined by injecting standard solutions containing between 0.001 and 10 mg/L (five data points in triplicate) *N*-lactoyl ethanolamine. R^2 values of the calibration plots were between 0.9996 and 0.9998 in all cases and LODs were found to be 3 μg/L in SIM and 2 mg/L in SRM mode. This external calibration plot was used for the quantification of *N*-lactoyl ethanolamine by SRM in AM mode. The concentration was 4 ppm in the Burgenland wine fraction and 50 ppm in the Ilbesheimer wine fraction, which were obtained by preparative HPLC. Therefore in the wines, the concentrations were 0.4 and 2.5 ppm, respectively.

4 Discussion

The taste-modulating flavor ingredient *N*-lactoyl ethanolamine was successfully isolated and determined in the two

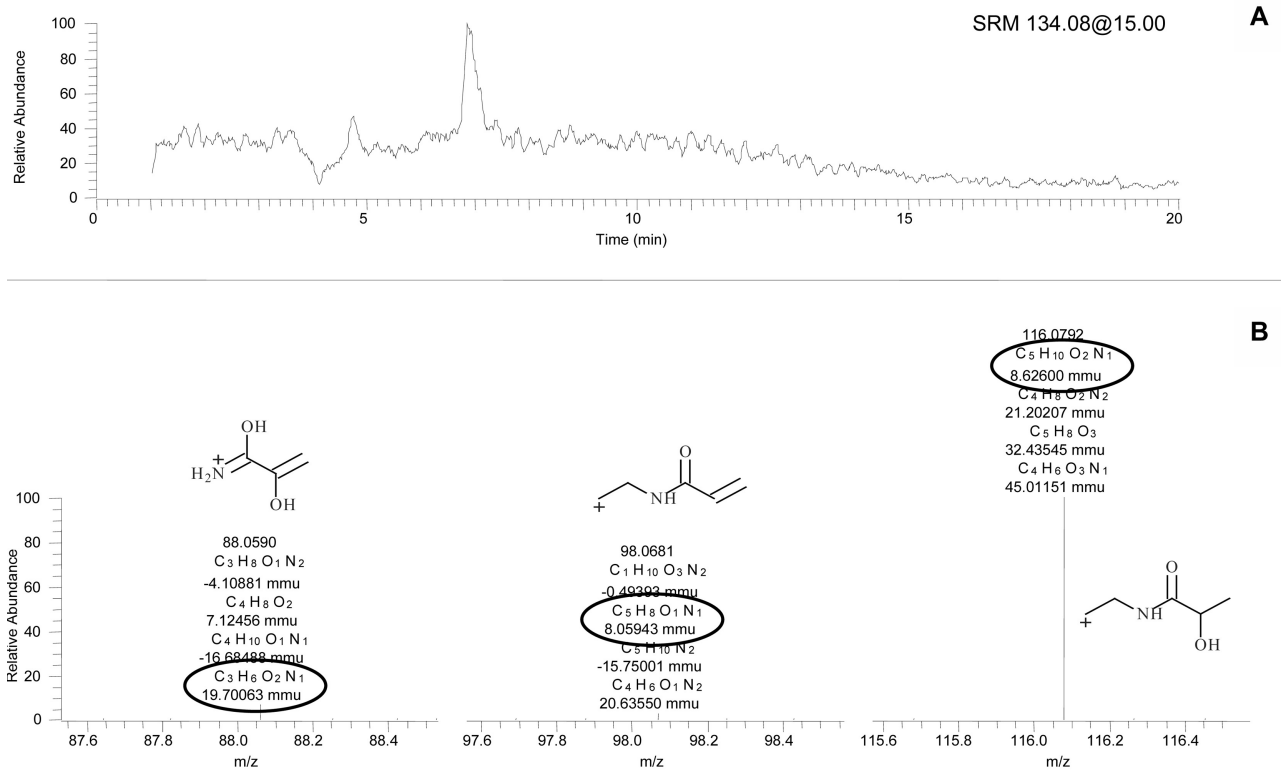

Figure 5. Selected reaction monitoring (SRM) of *m/z* 134.08 in Burgenland wine. A, reconstituted LC-MS/MS chromatogram of the three target transitions *m/z* 134 → 88, *m/z* 134 → 98, *m/z* 134 → 116; B, SRM spectrum of three selected fragments of *N*-lactoyl ethanolamine with accurate masses, possible elemental compositions, and mass accuracy in milli mass units (mmu). Collision energy, 15 eV.

Beerenauslese wines. It was shown that preparative LC is an efficient one-step approach to isolate and concentrate trace compounds out of aqueous matrices. AM determination of three characteristic MS/MS fragments of *N*-lactoyl ethanolamine was achieved between 8 and 20 mDa for Burgenland wine and between 8 and 28 mDa Ilbesheimer wine. In the wines, the concentrations of the target compound were 0.4 and 2.5 mg/L, respectively. The LODs for LC-MS were found to be 3 μg/L in SIM and 2 mg/L in SRM mode.

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